

10/509036

PCT/AU03/00388



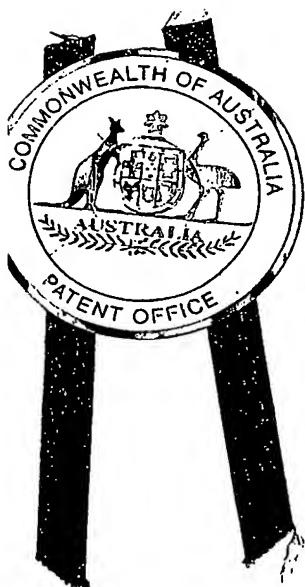
REC'D 25 APR 2003
WIPO PCT

Patent Office
Canberra

I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003900230 for a patent by MEDVET SCIENCE PTY.LTD. as filed on 21 January 2003.

WITNESS my hand this
Eleventh day of April 2003

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES



**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Regulation 3.

Medvet Science Pty. Ltd.

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A method of modulating cellular activity - V."

The invention is described in the following statement:

A METHOD OF MODULATING CELLULAR ACTIVITY - V

FIELD OF THE INVENTION

5 The present invention relates generally to a method of modulating cellular activity and to agents for use therein. More particularly, the present invention provides a method of modulating cellular activity by modulating phosphorylation of sphingosine kinase and, thereby, its activation. In a related aspect, the present invention provides a method of modulating sphingosine kinase functional activity via modulation of its phosphorylation

10 10 and agents for use therein. The present invention still further extends to sphingosine kinase variants and to derivatives, analogues, chemical equivalents and mimetics thereof exhibiting reduced and/or ablated capacity to undergo phosphorylation. The method and molecules of the present invention are useful, *inter alia*, in the treatment and/or prophylaxis of conditions characterised by aberrant, unwanted or otherwise inappropriate

15 15 cellular and/or sphingosine kinase functional activity. The present invention is further directed to methods for identifying and/or designing agents capable of modulating sphingosine kinase phosphorylation.

BACKGROUND OF THE INVENTION

20 Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

25 The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

30 Sphingosine kinase catalyses the formation of sphingosine 1-phosphate (S1P), a lipid messenger that plays important roles in a wide variety of mammalian cellular processes (Pyne *et al.* 2000; Spiegel 1999). S1P is mitogenic in various cell types and triggers a diverse range of important regulatory pathways including; mobilisation of intracellular

calcium by an inositol triphosphate independent pathway (Mattie *et al.* 1994), activation of phospholipase D (Desai *et al.* 1992), inhibition of c-Jun N-terminal kinase (JNK) (Cuvilliver *et al.* 1998), inhibition of caspases (Cuvilliver *et al.* 1998), adhesion molecule expression (Xia *et al.* 1998), and stimulation of DNA binding activity of NF- κ B (Xia *et al.* 5 2002) and transcription factor activator protein-1 (AP-1) (Su *et al.* 1994).

In addition to its role in cellular proliferation and survival, S1P appears to have other functions in the cell. For example, recent studies have shown that S1P is an obligatory signalling intermediate in adhesion molecule expression of vascular endothelial cells (Xia 10 *et al.* 1998), suggesting a likely role in inflammation and atherosclerosis.

Cellular levels of S1P are largely mediated by the activity of sphingosine kinase, and to a lesser extent by its degradation by S1P lyase (Van Veldhoven *et al.* 2000) and S1P phosphatase (Mandala *et al.* 2000) activities. Basal levels of S1P in the cell are generally 15 low (Spiegel *et al.* 1998), but can increase rapidly and transiently when cells are exposed to various mitogenic agents. This response is a direct consequence of an increase in sphingosine kinase activity in the cytosol and can be prevented by the addition of sphingosine kinase inhibitors. This places sphingosine kinase, and its activation, in a central and obligatory role in mediating the observed effects attributed to S1P in the cell. 20 However, at present almost nothing is known of the mechanism(s) leading to sphingosine kinase activation.

Sphingosine kinase can be very rapidly activated by wide variety of cell agonists. While the response differs between cell types, these stimuli include TNF α (Xia *et al.* 1998); 25 Pitson *et al.* 2000) (Fig 1), platelet-derived growth factor (Olivera *et al.*, 1993), epidermal growth factor (Meyer zu Heringdorf *et al.* 1999), nerve growth factor (Rius *et al.*, 1997), vitamin D3 (Kleuser *et al.* 1998), phorbol esters (Pitson *et al.* 2000; Buehrer *et al.* 1996), acetylcholine (muscarinic agonists) (Meyer zu Heringdorf *et al.* 1998), and crosslinking of the immunoglobulin receptors Fc ϵ R1 (Choi *et al.* 1996) and Fc γ R1 (Melendez *et al.* 1998). 30 In all cases this sphingosine kinase activation increases the V_{max} of the reaction while leaving the substrate affinities (K_m) unaltered.

The molecular mechanisms that couple these agonists to activation of sphingosine kinase activity remain largely unknown. Accordingly, there is a need to elucidate these mechanisms and to develop methods of regulating cellular activities via regulation of the 5 sphingosine kinase signalling pathways.

In work leading up to the present invention, the inventors have determined that phosphorylation of sphingosine kinase is essential for its activation. Further the inventors have identified the phosphorylation sites on the sphingosine kinase molecule. Further, it 10 has been determined that phosphorylation of sphingosine kinase is performed by a proline-directed protein kinase, more particularly ERK2.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will

5 be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The subject specification contains nucleotide sequence information prepared using the programme PatentIn Version 3.1, presented herein after the bibliography. Each nucleotide 10 sequence is identified in the sequence listing by the numeric indicator <201> followed by the sequence identifier (eg. <210>1, <210>2, etc). The length, type of sequence (DNA, etc) and source organism for each nucleotide sequence is indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively.

Nucleotide sequences referred to in the specification are identified by the indicator SEQ ID

15 NO: followed by the sequence identifier (eg. SEQ ID NO:1, SEQ ID NO:2, etc.). The sequence identifier referred to in the specification correlates to the information provided in numeric indicator field <400> in the sequence listing, which is followed by the sequence identifier (eg. <400>1, <400>2, etc). That is SEQ ID NO:1 as detailed in the specification correlates to the sequence indicated as <400>1 in the sequence listing.

20 Specific mutations in amino acid sequence are represented herein as "Xaa₁nXaa₂" where Xaa₁ is the original amino acid residue before mutation, n is the residue number and Xaa₂ is the mutant amino acid. The abbreviation "Xaa" may be the three letter or single letter amino acid code. A mutation in single letter code is represented, for example, by X₁nX₂

25 where X₁ and X₂ are the same as Xaa₁ and Xaa₂ respectively. In terms of both the mutation and the human sphingosine kinase protein sequence in general, the amino acid residues for human sphingosine kinase are numbered with the residue serine(S) in the motif KTPASPVVVQ SEQ ID NO:1 being numbered 225.

30 One aspect of the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an

effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

5

Another aspect of the present invention provides a method of modulating human sphingosine kinase functional activity, said method comprising contacting said human sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said human sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said human sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said human sphingosine kinase activity.

10

Yet another aspect of the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of said sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰, wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said sphingosine kinase activity.

15

Still another aspect of the present invention is directed to a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate proline-directed protein kinase catalysed sphingosine kinase phosphorylation wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

20

25

30

In a related aspect the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with

an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase, which agent binds, links or otherwise associates with serine ²²⁵, wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

5 Yet another aspect of the present invention is directed to a method of modulating cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

10 A further aspect of the present invention is directed to a method of modulating cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of human sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

15 In another further aspect, the present invention is directed to a method of modulating human cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰

20 wherein inducing or otherwise agonising said phosphorylation up-regulates said human cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said human cellular activity.

30 Yet another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, said method comprising

administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular

5 activity.

In still another aspect, the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a human, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, said method comprising

10 administering to said human an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰ wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

15 Another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, wherein said agent modulates the phosphorylation of sphingosine kinase and

20 wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

In yet another further aspect, the present invention contemplates a pharmaceutical

25 composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. These agents are referred to as the active ingredients.

Yet another aspect of the present invention relates to the agent as hereinbefore defined,

30 when used in the method of the present invention.

A further aspect of the present invention provides a method for detecting an agent capable of modulating the phosphorylation of sphingosine kinase or its functional equivalent or derivative thereof said method comprising contacting a cell or extract thereof containing said sphingosine kinase and phosphorylation catalyst or its functional equivalent or derivative with a putative agent and detecting an altered expression phenotype associated with said phosphorylation.

Still another aspect of the present invention is directed to agents identified in accordance with the screening method defined herein and to said agents for use in the methods of the present invention. Said agent should be understood to extend to monoclonal antibodies which bind to the phosphorylation sites of sphingosine kinase, and in particular, amino acids S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ and T²⁵⁰, and most particularly S²²⁵.

Still a further aspect of the present invention is directed to sphingosine kinase variants comprising a mutation in a region of said sphingosine kinase which region comprises a phosphorylation site, wherein said variant exhibits ablated or reduced phosphorylation capacity relative to wild type sphingosine kinase or a derivative, homologue, analogue, chemical equivalent or pneumatic of said sphingosine kinase variant.

In another aspect there is provided a human sphingosine kinase variant comprising an amino acid sequence with a single or multiple amino acid substitution and/or deletion of amino acids S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ and/or T²⁵⁰ wherein said variant exhibits ablated or reduced phosphorylation capacity relative to wild-type sphingosine kinase or a derivative, homologue, analogue, chemical equivalent or mimetic of said sphingosine kinase variant.

In yet another aspect, the present invention extends to genetically modified animals, which animals have been modified to express a sphingosine kinase variant as hereinbefore defined.

Single and three letter abbreviations used throughout the specification are defined in Table 1.

TABLE 1
Single and three letter amino acid abbreviations

	5	Amino Acid	Three-letter Abbreviation	One-letter Symbol
		Alanine	Ala	A
		Arginine	Arg	R
		Asparagine	Asn	N
10	10	Aspartic acid	Asp	D
		Cysteine	Cys	C
		Glutamine	Gln	Q
		Glutamic acid	Glu	E
		Glycine	Gly	G
15	15	Histidine	His	H
		Isoleucine	Ile	I
		Leucine	Leu	L
		Lysine	Lys	K
		Methionine	Met	M
20	20	Phenylalanine	Phe	F
		Proline	Pro	P
		Serine	Ser	S
		Threonine	The	T
		Tryptophan	Trp	W
25	25	Tyrosine	Tyr	Y
		Valine	Val	V
		Any residue	Xaa	X

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the phosphorylation of hSK1 paralleling its activation. TNF α simulation of hSK1-transfected HEK293T cells resulted in an *in vivo* phosphorylation of hSK1 which was paralleled by an increase in sphingosine kinase activity in these cells.

5 **Figure 2** is an image of the inactivation and lack of phosphorylation, in response to TNF α , of a mutant which is unable to bind TRAF2. Unlike wild-type hSK1, hSK^{TB2}, a hSK1 mutant defective in its ability to bind TRAF2 (Xia *et al.* 2002) is neither activated nor phosphorylated in response to TNF α treatment of HEK293T cells. In contrast, PMA treatment of these cells results in both phosphorylation and activation of wild-type hSK1 and hSK^{TB2}.

10 **Figure 3** is a schematic representation of the prediction of phosphorylated amino acids in hSK1 using NetPhos. Circled residues represent those amino acids predicted to be phosphorylated in hSK1 (and its mouse and monkey homologues) by the NetPhos program.

15 **Figure 4** is an image of the ablation of phosphorylation of hSK1 expressing an Ser²²⁵ \rightarrow Ala mutation. Metabolic labelling of HEK293T cells transfected with wild-type hSK1, hSK^{S148A}, hSK^{S181A}, hSK^{Y184A}, hSK^{S225A}, hSK^{T250A}, or empty vector and either untreated or treated with PMA showed that only the Ser²²⁵ \rightarrow Ala mutation ablated phosphorylation of hSK1.

20 **Figure 5** is an image of the phosphorylation of hSK1, expressing an Ser²²⁵ \rightarrow Ala mutation, at adjacent sites. Metabolic labelling of HEK293T cells transfected with wild-type hSK1, hSK^{S220A}, hSK^{S225A}, or hSK^{T222A} and either untreated or treated with PMA again showed that only the Ser²²⁵ \rightarrow Ala mutation ablated phosphorylation of hSK1. This 25 indicated that the Ser²²⁵ \rightarrow Ala mutation directly effects phosphorylation of hSK1 at Ser²²⁵ rather than indirectly effecting phosphorylation at adjacent sites (i.e. Ser²²⁰ and Thr²²²)

Figure 6 is a graphical representation of the inactivation of hSK^{S255A} by TNF α or PMA. Unlike wild-type hSK1-transfected HEK293T cells, hSK^{S255A}-transfected HEK293T cells show no increase in sphingosine kinase activity following treatment with TNF α or PMA.

5

Figure 7 is an image of the *in vitro* phosphorylation of hSK1. *In vitro* phosphorylation of purified recombinant hSK1 was examined with ERK1, ERK2 and CDK2. Quantitation of the specific activity of phosphorylation showed ERK2 phosphorylated purified recombinant hSK1 with greatest efficiency.

10

Figure 8 is an image of the *in vitro* phosphorylation of hSK1 by ERK2 occurring only at Ser²²⁵. *In vitro* phosphorylation of purified recombinant wild-type hSK1, hSK^{S225A}, hSK^{S148A} and hSK^{T222A} was examined with ERK2. Only hSK^{S225A} was not phosphorylated by ERK2 indicating that ERK2 specifically phosphorylates hSK1 at Ser²²⁵.

15

Figure 9A is an image of the increase in hSK1 phosphorylation being blocked by an ERK1/2 pathway inhibitor (PD98059) but not by a CDK inhibitor (Olomoucine). Treatment of hSK1-transfected HEK293T cells with 50 μ M PD98059 did not effect basal hSK1 phosphorylation, but blocked increases in hSK1 phosphorylation following TNF α or PMA treatment. In contrast, 20 μ M Olomoucine appeared to stimulate basal hSK1 phosphorylation which was further increased by PMA treatment.

20

Figure 9B is an image of the immunoprecipitation of ERK1/2 with hSK1. HEK293T cells overexpressing either wild type hSK1 or hSK1^{TB2} were treated with TNF α or PMA for 30 min, harvested and lysed as previously described (Pitson et al., 2000b). The clarified cell lysates were immunoprecipitated with M2 anti-FLAG antibody, subjected to SDS-PAGE and the protein complexes probed by Western blot for hSK1 and ERK1/2 using M2 anti-FLAG and anti-ERK1/2 antibodies, respectively.

Figure 10A is a graphical representation which indicates that hSK1 is directly activated by *in vitro* phosphorylation of Ser²²⁵ with ERK2. Data show sphingosine kinase activity (k_{cat}) of hSK1 prior to and following *in vitro* phosphorylation at Ser²²⁵ by ERK2. Values are corrected for the proportion of hSK1 phosphorylated in the assay mix.

5

Figure 10B is a graphical representation of the substrate kinetics with ATP of *in vitro* phosphorylated hSK1. The data indicate that phosphorylation of hSK1 at Ser²²⁵ results in a 13.6-fold increase in k_{cat} (93 s⁻¹ to 1265 s⁻¹). K_M values for ATP were 81 ± 12 μM and 56 ± 8 μM for non-phosphorylated and phosphorylated hSK1, respectively.

10

Figure 10C is a graphical representation of the substrate kinetics with sphingosine of *in vitro* phosphorylated hSK1. The data indicate that phosphorylation of hSK1 at Ser²²⁵ results in a 13.6-fold increase in k_{cat} (93 s⁻¹ to 1265 s⁻¹). K_M values for sphingosine were 15 ± 4 μM and 13 ± 3 μM for non-phosphorylated and phosphorylated hSK1, respectively.

15

Figure 11 is a graphical representation demonstrating that a Ser²²⁵ → Glu mutation in hSK1 does not create a constitutively activated hSK1. hSK^{WT} and hSK^{S225E} were expressed in HEK293T cells and the resultant sphingosine kinase activity in cell lysates quantitated with respect to protein expression levels.

20

Figure 12 is a schematic representation of the targets for sphingosine kinase antagonists.

25

Figure 13 is a graphical representation of an ELISA of phospho-hSK antiserum. Crude (open symbols) and affinity purified (closed symbols) antiserum from rabbits injected with a KLH-conjugated phosphopeptide designed around Ser²²⁵ of hSK1 was analysed by ELISA with the phosphopeptide (circles) and corresponding non-phosphorylated peptide (squares).

30

Figure 14 is an image illustrating that the phospho-hSK1 antiserum specifically reacts with phosphorylated hSK1 in Western blot analysis. Western blot analysis of hSK1 with the affinity purified phospho-hSK1 antiserum. Lane 1, recombinant hSK1; Lane 2,

recombinant hSK1 phosphorylated *in vitro* by ERK2; Lane 3, recombinant hSK1 phosphorylated *in vitro* by ERK2 and subsequently dephosphorylated by alkaline phosphatase.

5 **Figure 15** is an image illustrating that the phosph-hSK1 antiserum allows phosphorylation of *in vivo* hSK1 to be followed. HEK293T cells were transfected with wild-type hSK1 or hSK1^{S225A} and treated with TNF α or PMA, harvested and analysed by Western blot with the phospho-hSK1 antiserum and anti-FLAG antibody.

10 **Figure 16** is a graphical representation of the proliferation of HEK293T cells in response to overexpression of wild type hSK1 and hSK1^{S225A}. Cells transfected with wild type hSK1 (triangles), hSK1^{S225A} (diamonds), or empty vector (circles) were grown in DMEM media containing either 5 % foetal calf serum (FCS), 1 % FCS or no serum (0.1 % BSA). Cell numbers were determined using the MTT assay.

15 **Figure 17** is a graphical representation of the transformation of NIH3T3 cells in response to overexpression of wild type hSK1 and hSK1^{S225A}. (A) Panels on the left show colony formation in soft agar of NIH3T3 cells transfected with either empty vector, wild type hSK1 or hSK1^{S225A}. Panels on the right show colony formation in soft agar of NIH3T3

20 cells co-transfected with constitutively active V12-RAs and either empty vector, wild type hSK1 or hSK1^{S225A}. (B) Quantitation of colonies formed.

Figure 18 shows that phosphorylation of hSK1 leads to its translocation to membranes and increased intracellular and extracellular sphingosine 1-phosphate levels. **a**, Translocation of hSK1 from the cytosol to membranes is dependent on its phosphorylation. Cell lysates were fractionated into cytosol and membranes, and probed via Western blot for total hSK1 (with anti-FLAG) and phospho-hSK1. **b**, Intracellular and extracellular S1P levels were determined in HEK293T cells transiently transfected with empty vector, or plasmids

- 14 -

encoding for wild type hSK1 and hSK1^{S225A} following TNF α and PMA treatment, and in the presence or absence of the ERK1/2 pathway inhibitor U0126.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the determination that sphingosine kinase activation is mediated by a phosphorylation event and, further, the identification of the 5 sphingosine kinase phosphorylation site itself. The inventors have still further determined that sphingosine kinase phosphorylation can be mediated by members of the proline-directed protein kinase family and, most preferably, ERK2. These determinations now permit the rational design of therapeutic and/or prophylactic methods for treating conditions, such as those characterised by aberrant or unwanted cellular activity and/or 10 sphingosine kinase functional activity. Further, there is facilitated the identification and/or design of agents which specifically modulate sphingosine kinase phosphorylation.

Accordingly, one aspect of the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine 15 kinase with an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

20 Reference to "sphingosine kinase functional activity" should be understood as a reference to any one or more of the activities which sphingosine kinase can perform, for example, its activity as a key regulatory enzyme in the functioning of the sphingosine kinase signalling pathway. In this regard, it is thought that sphingosine kinase is central to the generation of 25 sphingosine-1-phosphate during activation of this pathway. It should be understood that modulation of sphingosine kinase functional activity encompasses both up and down-regulation of any one or more of the functional activities attributable to sphingosine kinase, such as the induction or cessation of a given activity or a change to the level or degree of any given activity. Without limiting the invention to any one theory or mode of action, 30 sphingosine kinase is thought to exhibit two levels of catalytic activity. At the first level, sphingosine kinase exhibits baseline catalytic activity. At the second level, sphingosine

kinase exhibiting baseline activity can be activated such that the Vmax of the enzyme is increased. In the context of the preferred embodiment of the present invention, the ablation or reduction of sphingosine kinase catalytic activity will be achieved where the baseline activity and/or the activation of sphingosine kinase beyond that of baseline 5 activity is ablated or reduced. Preferably, activation is ablated or reduced and even more preferably activation is ablated.

In a preferred embodiment, antagonising said phosphorylation prevents activation of sphingosine kinase and agonising said phosphorylation results in activation of said 10 sphingosine kinase.

Reference to "sphingosine kinase" should be understood to include reference to all forms of sphingosine kinase protein or derivates, homologues, analogues, equivalents or mimetics thereof. In this regard, "sphingosine kinase" should be understood as being a 15 molecule which is, *inter alia*, involved in the generation of sphingosine-1-phosphate during activation of the sphingosine kinase signalling pathway. This includes, for example, all protein forms of sphingosine kinase or its functional derivatives, homologues, analogues, equivalents or mimetics thereof, including, for example, any isoforms which arise from alternative splicing of sphingosine kinase mRNA or allelic or polymorphic 20 variants of sphingosine kinase. Preferably, said sphingosine kinase is human sphingosine kinase.

Accordingly, the present invention provides a method of modulating human sphingosine kinase functional activity, said method comprising contacting said human sphingosine 25 kinase with an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said human sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said human sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said human sphingosine kinase activity.

Reference to "phosphorylation" should be understood as a reference to the addition of a phosphate group to the hydroxyl groups on proteins, in particular to the side chains of the amino acids serine, threonine or tyrosine. Without limiting the present invention to any one theory or mode of action, this process of phosphorylating proteins is normally

5 catalysed by a protein kinase (such molecules herein being referred to as "phosphorylation catalysts"), often a specific protein kinase, with ATP acting as the phosphate donor. The phosphorylation of proteins is generally found to regulate the activity of the subject protein.

10 As detailed hereinbefore, the phosphorylation of a protein is an event which occurs in the context of specific amino acid residues of a subject protein. In this regard, the inventors have identified S^{148} , S^{181} , Y^{184} , S^{225} and T^{250} as being the amino acid residues which are relevant to phosphorylation of the sphingosine kinase molecule, in general. However, in terms of the human sphingosine kinase molecule, the inventors have still further identified

15 S^{225} as the primary physiological phosphorylation site of this molecule.

Accordingly, in a preferred embodiment, the present invention provides a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions

20 sufficient to modulate the phosphorylation of said sphingosine kinase at one or more of S^{148} , S^{181} , Y^{184} , S^{225} or T^{250} , wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said sphingosine kinase activity.

25 In a most preferred embodiment, said sphingosine kinase is human sphingosine kinase and said phosphorylation modulation occurs at the amino acid residue S^{225} .

Reference to "modulating" either sphingosine kinase functional activity or a phosphorylation event should be understood as a reference to up-regulating or down-regulating the subject functional activity or phosphorylation event. Reference to up-regulating and down-regulating in this regard should be understood to include both

increasing or decreasing the level, degree or rate at which the functional activity or phosphorylation event occurs, in addition to including reference to inducing or ablating the subject functional activity or phosphorylation event. Accordingly, the agent which is utilised in accordance with the method of the present invention may be an agent which 5 induces the activity/event, agonises an activity or event which has already undergone onset, antagonises a pre-existing activity or event or entirely ablates such an activity or event.

Accordingly, reference to "inducing or otherwise agonising" phosphorylation should be 10 understood as a reference to:

- (i) inducing the phosphorylation of sphingosine kinase, for example, inducing the interaction of sphingosine kinase with a proline-directed protein kinase, such as ERK2, which effects sphingosine kinase phosphorylation; or
- 15 (ii) up-regulating, enhancing or otherwise agonising an existing phosphorylation event, for example, increasing the affinity of or otherwise stabilising the interaction of sphingosine kinase with a phosphorylating molecule.

20 Conversely, "inhibiting or otherwise antagonising" phosphorylation should be understood as a reference to:

- (i) preventing the interaction of sphingosine kinase with a phosphorylating molecule; or
- 25 (ii) antagonising an existing interaction between sphingosine kinase and a phosphorylating molecule such that the phosphorylation of sphingosine kinase is rendered ineffective or less effective.

It should be understood that modulation (either in the sense of up-regulation or down-regulation) of the phosphorylation of sphingosine kinase may be partial or complete. 30

Partial modulation occurs where only some of the sphingosine kinase phosphorylation events which would normally occur in a given cell or on a given molecule are affected by the method of the present invention while complete modulation occurs where all sphingosine kinase phosphorylation events are modulated.

5

Modulation of the phosphorylation of sphingosine kinase may be achieved by any one of a number of techniques including, but not limited to:

- 10 (i) introducing into a cell a proteinaceous or non-proteinaceous agent which antagonises the interaction between sphingosine kinase and a phosphorylation catalyst;
- (ii) introducing into a cell a proteinaceous or non-proteinaceous agent which agonises the interaction between sphingosine kinase and a phosphorylation catalyst;
- 15 (iii) introducing into a cell a proteinaceous or non-proteinaceous agent which acts as a phosphorylation catalyst;
- (iv) introducing into a cell a nucleic acid molecule which encodes an agent which acts as a phosphorylation catalyst.

20 Preferably, said phosphorylation occurs at Ser²²⁵ of sphingosine kinase, and even more preferably, at Ser²²⁵ of human sphingosine kinase.

25 Reference to "agent" should be understood as a reference to any proteinaceous or non-proteinaceous molecule which modulates (i.e. up-regulates or down-regulates) the interaction of sphingosine kinase with a phosphorylation catalyst, for example, the molecules detailed in points (i) - (ii), above. "Agent" should also be understood to extend to molecules which, of themselves, function as a phosphorylation catalyst, for example, the molecules detailed in (iii) - (iv), above. The subject agent may be linked, bound or

30

otherwise associated with any proteinaceous or non-proteinaceous molecule. For example, it may be associated with a molecule which permits targeting to a localised region.

Said proteinaceous molecule may be derived from natural, recombinant or synthetic sources including fusion proteins or following, for example, natural product screening.

5 Said non-proteinaceous molecule may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. For example, the present invention contemplates chemical analogues of sphingosine kinase or phosphorylation catalyst, such as a kinase molecule (for example, a proline-directed protein kinase such as ERK2), capable of acting as agonists or antagonists of sphingosine kinase phosphorylation. Chemical agonists may not necessarily be derived from sphingosine kinase or the phosphorylation catalyst but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of sphingosine kinase or phosphorylation catalyst.

10 Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing sphingosine kinase phosphorylation. Antagonists include antibodies (such as monoclonal and polyclonal antibodies) specific for sphingosine kinase or the phosphorylation catalyst, or parts of said sphingosine kinase, and antisense nucleic acids which prevent transcription or translation of genes or mRNA in the subject cells. Modulation of expression may also

15 be achieved utilising antigens, RNA, ribosomes, DNAzymes, RNA aptamers, antibodies or molecules suitable for use in co-suppression. Screening methods suitable for use in identifying such molecules are described in more detail hereinafter.

Said proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the interaction of sphingosine kinase with a phosphorylating molecule (such as a phosphorylating kinase). Said molecule acts directly if it associates with the sphingosine kinase or the phosphorylation catalyst. Said molecule acts indirectly if it associates with a molecule other than the sphingosine kinase or phosphorylation catalyst, which other molecule either directly or indirectly modulates the interaction of sphingosine kinase with the phosphorylation catalyst. Accordingly, the method of the present invention

encompasses regulation of sphingosine kinase phosphorylation via the induction of a cascade of regulatory steps.

It should be understood that reference to "phosphorylation catalyst" is intended to
5 encompass both molecules which catalyse the transfer of phosphate from a molecule such
as ATP to sphingosine kinase. However, it should also be understood to encompass
reference to molecules which otherwise achieve the phosphorylation of sphingosine kinase,
such as molecules which are able to transfer phosphate groups from molecules other than
ATP or which can directly transfer a phosphate group from the phosphorylation catalyst
10 itself to the sphingosine kinase molecule.

"Derivatives" include fragments, parts, portions, mutants, variants and mimetics from
natural, synthetic or recombinant sources including fusion proteins. Parts or fragments
include, for example, active regions of sphingosine kinase or the phosphorylation catalyst.
15 Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino
acid insertional derivatives include amino and/or carboxylic terminal fusions as well as
intrasequence insertions of single or multiple amino acids. Insertional amino acid
sequence variants are those in which one or more amino acid residues are introduced into a
predetermined site in the protein although random insertion is also possible with suitable
20 screening of the resulting product. Deletional variants are characterized by the removal of
one or more amino acids from the sequence. Substitutional amino acid variants are those
in which at least one residue in the sequence has been removed and a different residue
inserted in its place. An example of substitutional amino acid variants are conservative
amino acid substitutions. Conservative amino acid substitutions typically include
25 substitutions within the following groups: glycine and alanine; valine, isoleucine and
leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine;
lysine and arginine; and phenylalanine and tyrosine. Additions to amino acid sequences
include fusions with other peptides, polypeptides or proteins.
30 Reference to "homologues" should be understood as a reference to nucleic acid molecules
or proteins derived from alternative species.

Equivalents of nucleic acid or protein molecules should be understood as molecules exhibiting any one or more of the functional activities of these molecules and may be derived from any source such as being chemically synthesized or identified via screening processes such as natural product screening.

5

The derivatives include fragments having particular epitopes or parts of the entire protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules.

10

10 Analogues contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues.

15

15 Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules. Derivatives of nucleic acid sequences

20

20 also include degenerate variants.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH₄; amidination with methylacetimidate;

25

25 acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with NaBH₄.

30

30 The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with

5 iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline
10 pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with
15 tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

20 Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl
25 alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated herein is shown in Table 2.

TABLE 2

5	Non-conventional amino acid	Code	Non-conventional amino acid	Code
	α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
	α -amino- α -methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
10	aminocyclopropane- carboxylate	Cpro	L-N-methyleasparagine	Nmasn
	aminoisobutyric acid	Aib	L-N-methyleaspartic acid	Nmasp
	aminonorbornyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
15	cyclohexylalanine	Chexa	L-N-methylglutamine	Nmgln
	cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
	D-alanine	Dal	L-N-methylhistidine	Nmhis
	D-arginine	Darg	L-N-methylisoleucine	Nmile
	D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
20	D-cysteine	Dcys	L-N-methyllysine	Nmlys
	D-glutamine	Dgln	L-N-methylmethionine	Nmmet
	D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
	D-histidine	Dhis	L-N-methylnorvaline	Nmnva
	D-isoleucine	Dile	L-N-methylornithine	Nmorn
25	D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
	D-lysine	Dlys	L-N-methylproline	Nmpro
	D-methionine	Dmet	L-N-methylserine	Nmser
	D-ornithine	Dorn	L-N-methylthreonine	Nmthr
	D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
30	D-proline	Dpro	L-N-methyltyrosine	Nmtyr
	D-serine	Dser	L-N-methylvaline	Nmval
	D-threonine	Dthr	L-N-methylethylglycine	Nmetg
			L-N-methyl-t-butylglycine	Nmtbug
			L-norleucine	Nle

	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
	D-valine	Dval	α -methyl- α -aminobutyrate	Mgabu
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
5	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
30	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu

	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
5	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
10	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
15	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
20	L- α -methylleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
25	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
	N-(N-(2,2-diphenylethyl)carbamylmethyl)glycine	Nnbhm.	N-(N-(3,3-diphenylpropyl)carbamylmethyl)glycine	Nnbhe
	1-carboxy-1-(2,2-diphenyl-Nmboethylamino)cyclopropane			
30				

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety.

Without limiting the present invention to any one theory or mode of action, the inventors have utilised alanine mutagenesis to determine that serine²²⁵ is the primary physiologic phosphorylation site on human sphingosine kinase 1. Further, it has been determined that 10 the kinases of the proline-directed protein kinase family are able to phosphorylate human sphingosine kinase. Specifically, ERK1, ERK2 and CDK2 phosphorylate human sphingosine kinase 1 to varying degrees of efficiency. ERK2, however, has been demonstrated to show the greatest efficiency in human sphingosine kinase phosphorylation.

15 Accordingly, in a most preferred embodiment the present invention is directed to a method of modulating sphingosine kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate proline-directed protein kinase catalysed sphingosine 20 kinase phosphorylation wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

Most preferably, said proline directed protein kinase is ERK1, ERK2 or CDK2 and, even 25 more particularly, ERK2.

Most preferably, said phosphorylation is modulated at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰ and most particularly at S²²⁵.

30 Reference to proline-directed protein kinase molecules, such as ERK1, ERK2 and CDK2, and any other phosphorylation catalyst should be understood to encompass reference to

derivatives, homologues, analogues, chemical equivalents and mimetics of these molecules.

In a related aspect the present invention provides a method of modulating sphingosine

5 kinase functional activity, said method comprising contacting said sphingosine kinase with an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of said sphingosine kinase, which agent binds, links or otherwise associates with serine ²²⁵, wherein inducing or otherwise agonising said phosphorylation up-regulates said sphingosine kinase activity and inhibiting or otherwise antagonising said phosphorylation down-regulates sphingosine kinase activity.

In a most preferred embodiment, said modulation of sphingosine kinase activity is down-regulation.

15 Since sphingosine kinase is a molecule which is central to the functioning of an intracellular signalling pathway, the method of the present invention provides a means of modulating cellular activity which is regulated or controlled by sphingosine kinase signalling. For example, the sphingosine kinase signalling pathway is known to regulate cellular activities, such as those which lead to inflammation, cellular transformation, 20 apoptosis, cell proliferation, up-regulation of the production of inflammatory mediators such as cytokines, chemokines, eNOS and up-regulation of adhesion molecule expression. Said up-regulation may be induced by a number of stimuli including, for example, inflammatory cytokines such as tumour necrosis factor α and interleukin 1, endotoxin, oxidised or modified lipids, radiation or tissue injury. In this regard, reference to 25 "modulating cellular activity" is a reference to up-regulating, down-regulating or otherwise altering any one or more of the activities which a cell is capable of performing pursuant to sphingosine kinase signalling such as, but not limited, one or more of chemokine production, cytokine production, nitric oxide synthesis, adhesion molecule expression and production of other inflammatory modulators. Although the preferred method is to down- 30 regulate sphingosine kinase activity, thereby down-regulating unwanted cellular activity,

the present invention should nevertheless be understood to encompass up-regulating of cellular activity, which may be desirable in certain circumstances.

Accordingly, yet another aspect of the present invention is directed to a method of 5 modulating cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

10 Preferably, the present invention is directed to a method of modulating cellular activity, said method comprising contacting said cell with an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of human sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up- 15 regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

In a most preferred embodiment the present invention is directed to a method of modulating human cellular activity, said method comprising contacting said cell with an 20 effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰ wherein inducing or otherwise agonising said phosphorylation up-regulates said human cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said human cellular activity.

25 Most preferably, said phosphorylation modulation occurs at amino acid residue S²²⁵ and said modulation is down-regulation.

30 A further aspect of the present invention relates to the use of the invention in relation to the treatment and/or prophylaxis of disease conditions. Without limiting the present invention to any one theory or mode of action, the broad range of cellular functional activities which

are regulated via the sphingosine kinase signalling pathway renders the regulation of sphingosine kinase functioning an integral component of every aspect of both healthy and disease state physiological processes. Accordingly, the method of the present invention provides a valuable tool for modulating aberrant or otherwise unwanted cellular functional activity which is regulated via the sphingosine kinase signalling pathway.

Accordingly, yet another aspect of the present invention is directed to a method for the treatment and/or prophylaxis of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, said method comprising 10 administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate phosphorylation of sphingosine kinase wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

15 Preferably, said phosphorylation event is phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰.

In a most preferred embodiment, the present invention is directed to a method for the 20 treatment and/or prophylaxis of a condition in a human, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, said method comprising administering to said human an effective amount of an agent for a time and under conditions sufficient to modulate the phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰ wherein inducing or otherwise agonising said 25 phosphorylation up-regulates said cellular activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

Most preferably, said phosphorylation event is phosphorylation of sphingosine kinase at S²²⁵ and still more preferably, said modulation is down-regulation:

Reference to "aberrant, unwanted or otherwise inappropriate" cellular activity should be understood to be understood as a reference to overactive cellular activity, to physiological normal cellular activity which is inappropriate in that it is unwanted or to insufficient cellular activity. This definition applies in an analogous manner in relation to "aberrant, 5 unwanted or otherwise, inappropriate" sphingosine kinase activation. For example, TNF production during tumour cell growth has been shown to support cellular proliferation and to provide anti-apoptotic characteristics to the neo plastic cells. Accordingly, to the extent that a cell is neoplastic, it is desirable that the promotion of cellular proliferation and anti-apoptotic characteristics be down-regulated. Similarly, diseases which are characterised 10 by inflammation, such as rheumatoid arthritis, are known to involve cellular activation by cytokines such as TNF, leading to the synthesis and secretion of inflammatory mediators. In such situations, it is also desirable to down-regulate such activity. In other situations, it may be desirable to agonise or otherwise induce sphingosine kinase phosphorylation in order to stimulate cellular proliferation.

15 In accordance with the previous aspects of the present invention, the subject agent is preferably PD98059 or derivative, analogue, chemical equivalent or mimetic thereof.

The term "mammal" as used herein includes humans, primates, livestock animals (eg. 20 sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer). Preferably, the mammal is human or a laboratory test animal. Even more preferably, the mammal is a human.

25 An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the degree of protection desired, the formulation of the 30 composition, the assessment of the medical situation, and other relevant factors. It is

expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest 5 context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as 10 reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

The present invention further contemplates a combination of therapies, such as the 15 administration of the agent together with subjection of the mammal to other agents, drugs or treatments which may be useful in relation to the treatment of the subject condition such as cytotoxic agents or radiotherapy in the treatment of cancer.

Administration of the modulatory agent, in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical 20 composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage 25 regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

30 The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous,

intradermal or suppository routes or implanting (e.g. using slow release molecules). The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such

5 acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

10

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraocularly, intrathecally, intracerebrally, intranasally, infusion, orally, rectally, via IV drip patch and implant.

15

In accordance with these methods, the agent defined in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the 20 same or different routes: For example, the subject agent may be administered together with an agonistic agent in order to enhance its effects. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

25

Another aspect of the present invention contemplates the use of an agent, as hereinbefore defined, in the manufacture of medicament for the treatment of a condition in a mammal, which condition is characterised by aberrant, unwanted or otherwise inappropriate cellular activity, wherein said agent modulates the phosphorylation of sphingosine kinase and 30 wherein inducing or otherwise agonising said phosphorylation up-regulates said cellular

activity and inhibiting or otherwise antagonising said phosphorylation down-regulates said cellular activity.

Preferably, said phosphorylation is phosphorylation of sphingosine kinase at one or more of S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ or T²⁵⁰.

Even more preferably, said mammal is a human, said phosphorylation is phosphorylation of sphingosine kinase at S²²⁵ and said modulation is down-regulation.

10 As detailed hereinbefore, and without limiting the present invention to any one theory or mode of action, it is thought that phosphorylation of sphingosine kinase activates sphingosine kinase exhibiting baseline activity such that the V_{max} of the enzyme is increased. Accordingly, screening individuals for the presence, absence or degree of sphingosine kinase phosphorylation provides a means for diagnosing, monitoring or 15 screening patients for conditions characterised by aberrantly activated sphingosine kinase activity and/or cellular activity. The present invention should therefore be understood to extend to such diagnostic assays.

In yet another further aspect, the present invention contemplates a pharmaceutical 20 composition comprising the modulatory agent as hereinbefore defined together with one or more pharmaceutically acceptable carriers and/or diluents. These agents are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions 25 (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion 30 medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and

vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens,

5 chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

10 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle 15 which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

20 When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the 25 active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The 30 amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the

present invention are prepared so that an oral dosage unit form contains between about 0.1 µg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed 5 hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, 10 in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of 15 course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The pharmaceutical composition may also comprise genetic molecules such as a vector 20 capable of transfecting target cells where the vector carries a nucleic acid molecule encoding a modulatory agent. The vector may, for example, be a viral vector.

Yet another aspect of the present invention relates to the agent as hereinbefore defined, 25 when used in the method of the present invention.

The present invention should also be understood to encompass a method for screening for agents which modulate the phosphorylation of sphingosine kinase, particularly at Ser²²⁵, such as agents which agonise or antagonise the interaction of sphingosine kinase with a phosphorylation catalyst (such as ERK2) or which themselves phosphorylate sphingosine 30 kinase, for example by functioning as a phosphorylation catalyst.

Screening for the modulatory agents hereinbefore defined can be achieved by any one of several suitable methods including, but in no way limited to, contacting a cell comprising sphingosine kinase and a phosphorylation catalyst (such as ERK2) with an agent and screening for the modulation of sphingosine kinase phosphorylation or modulation of the 5 activity or expression of a downstream sphingosine kinase cellular target such as NF- κ B. Detecting such modulation can be achieved utilising techniques such as Western blotting, electrophoretic mobility shift assays and/or the readout of reporters of sphingosine kinase activity such as luciferases, CAT and the like.

10 It should be understood that the sphingosine kinase or phosphorylation catalyst may be naturally occurring in the cell which is the subject of testing or the genes encoding them may have been transfected into a host cell for the purpose of testing. Further, the naturally occurring or transfected gene may be constitutively expressed - thereby providing a model useful for, *inter alia*, screening for agents which down-regulate sphingosine kinase 15 phosphorylation or the gene may require activation - thereby providing a model useful for, *inter alia*, screening for agents which modulate sphingosine kinase phosphorylation under certain stimulatory conditions. Further, to the extent that a sphingosine kinase nucleic acid molecule is transfected into a cell, that molecule may comprise the entire sphingosine kinase gene or it may merely comprise a portion of the gene such as a portion comprising 20 Ser²²⁵.

In another example, the subject of detection could be a downstream sphingosine kinase regulatory target, rather than sphingosine kinase itself, such as NF- κ B. Yet another example includes sphingosine kinase binding sites ligated to a minimal reporter. For 25 example, modulation of sphingosine kinase phosphorylation can be detected by screening for the modulation of the downstream signalling components of a TNF stimulated cell. Where the cell which is the subject of the screening system is a neoplastic cell, for example, modulation of sphingosine kinase phosphorylation could be detected by screening for the induction of apoptosis of that cell.

Accordingly, another aspect of the present invention provides a method for detecting an agent capable of modulating the phosphorylation of sphingosine kinase or its functional equivalent or derivative thereof said method comprising contacting a cell or extract thereof containing said sphingosine kinase and phosphorylation catalyst or its functional equivalent or derivative with a putative agent and detecting an altered expression phenotype associated with said phosphorylation.

Reference to "sphingosine kinase" and "phosphorylation catalyst" should be understood as a reference to either the sphingosine kinase or phosphorylation catalyst expression product or to a portion or fragment of the sphingosine kinase or phosphorylation catalyst, such as the Ser²²⁵ sphingosine kinase phosphorylation site. In this regard, the sphingosine kinase or phosphorylation catalyst expression product is expressed in a cell. The cell may be a host cell which has been transfected with the sphingosine kinase or phosphorylation catalyst nucleic acid molecule or it may be a cell which naturally contains the sphingosine kinase gene. Reference to "extract thereof" should be understood as a reference to a cell free transcription system.

Reference to detecting an "altered expression phenotype associated with said phosphorylation" should be understood as the detection of cellular changes associated with modulation of the phosphorylation of sphingosine kinase. These may be detectable, for example, as intracellular changes or changes observable extracellularly. For example, this includes, but is not limited to, detecting changes in downstream product levels or activities (e.g. NF- κ B).

Still another aspect of the present invention is directed to agents identified in accordance with the screening method defined herein and to said agents for use in the methods of the present invention. Said agent should be understood to extend to monoclonal antibodies which bind to the phosphorylation sites of sphingosine kinase, and in particular, amino acids S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ and T²⁵⁰, and most particularly S²²⁵.

Still a further aspect of the present invention is directed to sphingosine kinase variants comprising a mutation in a region of said sphingosine kinase which region comprises a phosphorylation site, wherein said variant exhibits ablated or reduced phosphorylation capacity relative to wild type sphingosine kinase or a derivative, homologue, analogue, 5 chemical equivalent or pneumatic of said sphingosine kinase variant.

The present invention also extends to variants which exhibit enhanced or up-regulated activity due to the nature of the mutation of an existing phosphorylation site or the incorporation of additional phosphorylation sites.

10 Reference to "mutation" should be understood as a reference to any change, alteration or other modification, whether occurring naturally or non-naturally, which modulates the capacity of said sphingosine kinase to undergo phosphorylation. Said modulation may be up-regulation or down-regulation. Although the present invention is preferably directed to variants which exhibit ablated activation capacity, it should be understood that the present 15 invention extends to the generation of variants which exhibit additional phosphorylation sites or an improved level of phosphorylation capacity of existing sites.

20 The change, alteration or other modification may take any form including, but not limited to, a structural modification (such an alteration in the secondary, tertiary or quaternary structure of the sphingosine kinase molecule), a molecular modification (such as an addition, substitution or deletion of one or more amino acids from the sphingosine kinase protein) or a chemical modification. The subject modification should also be understood to extend to the fusion, linking or binding of a proteinaceous or non-proteinaceous 25 molecule to the sphingosine kinase protein or to the nucleic acid molecule encoding a sphingosine kinase protein. It should also be understood that although it is necessary that the subject mutation is expressed by the sphingosine kinase expression product, the creation of the mutation may be achieved by any suitable means including either mutating a wild-type sphingosine kinase protein, synthesising a sphingosine kinase variant or 30 modifying a nucleic acid molecule encoding a wild-type sphingosine kinase protein such that the expression product of said mutated nucleic acid molecule is a sphingosine kinase

protein variant. Preferably, said mutation is a single or multiple amino acid sequence substitution, addition and/or deletion.

In accordance with this preferred embodiment there is provided a human sphingosine 5 kinase variant comprising an amino acid sequence with a single or multiple amino acid substitution and/or deletion of amino acids S¹⁴⁸, S¹⁸¹, Y¹⁸⁴, S²²⁵ and/or T²⁵⁰ wherein said variant exhibits ablated or reduced phosphorylation capacity relative to wild-type sphingosine kinase or a derivative, homologue, analogue, chemical equivalent or mimetic of said sphingosine kinase variant.

10 Preferably, amino acid is amino acid S²²⁵ and even more preferably said mutation is a Ser²²⁵ Ala substitution.

In terms of the present invention, reference to "wild-type" sphingosine kinase is a reference 15 to the forms of sphingosine kinase expressed by most individuals in a given population. There may be greater than one wild-type form of sphingosine kinase (for example due to allelic or isoform variation) and the level of phosphorylation exhibited by said wild-type sphingosine kinase molecules may fall within a range of levels. However, it should be understood that "wild-type" does not include reference to a naturally occurring form of 20 sphingosine kinase which cannot be phosphorylated. Such a variant form of sphingosine kinase may, in fact, constitute a naturally occurring mutant form of sphingosine kinase within the context of the present invention.

In yet another aspect, the present invention extends to genetically modified animals, which 25 animals have been modified to express a sphingosine kinase variant as hereinbefore defined.

The present invention is further described by reference to the following non-limiting description.

EXAMPLE 1

Methods

Generation of phospho-hSK1-specific polyclonal antibodies

5

Polyclonal antibodies were raised in rabbits against a phosphopeptide (CGSKTPApSPVVVQQ) designed from the hSK1 sequence around Ser²²⁵. Prior to injection into rabbits, the phosphopeptide was conjugated to maleimide activated keyhole limpet hemocyanin (Pierce) via its *N*-terminal cysteine. Antibodies active against the non-phosphorylated peptide were removed from the antiserum using SulfoLink™ beads (Pierce) to which the non-phosphorylated peptide (CGSKTPASPVVVQQ SEQ ID NO:2) was conjugated using the manufacturer's instructions.

Construction and expression of hSK mutants

15

Wild-type human SK1 (hSKWT) cDNA (Pitson *et al.* 2000) (Genbank accession number AF200328) was FLAG epitope tagged at the 3' end and subcloned into pALTER (Promega Inc., Madison, WI) site directed mutagenesis vector, as previously described (Pitson *et al.* 2000). Single-stranded DNA was prepared and used as template for oligonucleotide 20 directed mutagenesis as detailed in the manufacturer's protocol. The mutagenic oligonucleotides shown in Table 1 were designed to generate the required mutants which were subsequently sequenced to verify incorporation of the desired modification. The mutant cDNA were then subcloned into pcDNA3 (Invitrogen Corp., San Diego, CA) for transient transfection into HEK293T cells or pGEX-4T2 for expression as glutathione-*S*-transferase (GST)-fusion proteins in *E. coli*. hSK^{TB2} was generated as previously described 25 (Xia *et al.* 2002).

Cell Culture, transfection and cell fractionation

30 Human embryonic kidney cells (HEK293T, ATCC CRL-1573) were cultured on Dulbecco's modified Eagle's medium (CSL Biosciences, Parkville, Australia) containing

10% fetal calf serum, 2 mM glutamine, 0.2% (w/v) sodium bicarbonate, penicillin (1.2 mg/ml), and gentamycin (1.6 mg/ml). Transfections were performed using the calcium phosphate precipitation method. Cells were harvested 24 h after transfection and lysed by sonication (2 watts for 30 s at 4 °C) in lysis buffer A containing 50 mM Tris/HCl (pH 7.4), 5 10% (w/v) glycerol, 0.05% (w/v) Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 2 mM Na3VO4, 10 mM NaF, 1 mM EDTA and protease inhibitors (Complete™; Boehringer Mannheim). Activation of hSK1 was assessed by treatment of cells with either phorbol 12-myristate 13-acetate (PMA; Sigma) for 30 min, or tumor necrosis factor- α (TNF α ; R&D Systems Inc., Minneapolis, MN) for 10 min. For cell fractionation, HEK293T cells 10 were scraped into lysis buffer lacking Triton X-100, sonicated, and centrifuged at 1,000 x g for 10 min. The supernatants were then centrifuged at 100,000 x g for 60 min at 4 °C to yield membrane and cytosolic fractions. The membrane fraction was then resuspended in lysis buffer containing 0.8% Triton X-100, sonicated, left on ice for 30 min, and centrifuged at 10,000 x g for 10 min at 4 °C to remove Triton-insoluble material. Protein concentrations in 15 cell homogenates were determined with Coomassie Brilliant Blue (Sigma) reagent using BSA as standard.

Generation of recombinant proteins in E. coli

20 Recombinant proteins were generated of in *E. coli* and purified as previously described (Pitson *et al.* 2000).

Metabolic labelling of cells with ^{32}P

25 HEK293T cells were cultured and transfected as described above. The cultures, in 10 cm dishes, were incubated for 30 min with phosphate-free, serum-free Dulbecco's modified Eagle's medium (DMEM). This medium was then replaced with fresh phosphate-free, serum-free DMEM containing 500 μ Ci [^{32}P]orthophosphate and the cultures incubated for 3 hr. Cultures were then stimulated with 1 ng/ml TNF or 100 ng/ml PMA for 30 min. Cells 30 were then washed 3 times with cold PBS and scraped into 1.2 ml of lysis buffer B containing 50 mM Tris/HCl (pH 7.4), 1 % (w/v) Triton X-100, 1 % (w/v) deoxycholate,

0.1% SDS, 150 mM NaCl, 50 mM NaF, 10 mM sodium orthovanadate, 1 mM EDTA, 1 mM DTT, and protease inhibitors (Complete™). Cells were subjected to three freeze-thaw cycles to ensure complete disruption. The cell debris was then removed by centrifugation (13,000 g for 20 min at 4°C) and the supernatant incubated with 12 µg of M2 anti-FLAG antibody (Sigma) overnight with mixing at 4°C. The immunocomplexes were then collected with Protein A-Sepharose and washed four times with lysis buffer B. The immunoprecipitates were then subjected to SDS-PAGE on 12 % acrylamide gels, the gels dried and the phosphorylated hSK quantitated by Phosphorimager (Molecular Dynamics).

10 *In vitro phosphorylation of hSK1*

Purified recombinant hSK1 (1.5 µg) was incubated with recombinant ERK2 (0.1 µg; Upstate Biotechnology), 125 µM ATP and 5 µCi [γ ³²P]ATP in 20 mM MOPS (pH 7.2) containing 20 mM MgCl₂, 25 mM β -glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM DTT. The reaction was stopped after 20 min at 37°C by the addition of SDS-PAGE sample buffer, the mixture boiled for 5 min, subjected to SDS-PAGE on 12 % acrylamide gels, the gels dried, and the phosphorylated hSK1 quantitated by Phosphorimager.

20 *Sphingosine kinase assays*

SK activity was routinely determined using D-erythro-sphingosine (Biomol, Plymouth Meeting, PA) and [γ 32P]ATP (Geneworks, Adelaide, South Australia) as substrates, as described previously (Pitson *et al.* 2000). A unit (U) of SK activity is defined as the 25 amount of enzyme required to produce 1 pmol S1P/min.

S1P levels

To determine both intracellular and extracellular S1P levels HEK293T cells were metabolically labeled with [³²P]orthophosphate as described above. Cells were then 30 transferred to fresh phosphate-free DMEM and treated with 1 ng ml⁻¹ TNF α or 100 ng ml⁻¹ PMA for 30 min. To determine extracellular S1P release following stimulation, the media was

then removed, centrifuged at 1,000 x g, and 2.5 ml of the supernatant added to 2.5 ml chloroform, 2.5 ml methanol and 20 μ l conc. HCl. The organic phase was then dried under vacuum, resuspended in chloroform and S1P resolved by TLC on silica gel 60 with 1-butanol/ethanol/acetic acid/water (8:2:1:2, v/v). Intracellular S1P levels were determined by 5 harvesting the cells into 400 μ l methanol containing 25 μ l conc. HCl. Lipids were then extracted under alkaline conditions by the addition of 400 μ l chloroform, 400 μ l KCl and 40 μ l 10 3 M NaCl. The aqueous phase, containing S1P under these conditions, was then acidified through the addition of 50 μ l conc. HCl and re-extracted with 400 μ l chloroform. The organic phase organic phase was then dried under vacuum, resuspended in chloroform and S1P resolved by TLC as described above.

EXAMPLE 2

Results

15 *hSK1 is phosphorylated in HEK293 cells in response to cell agonists*

The *in vivo* phosphorylation of hSK1 in HEK293 cells in response to TNF α and phorbol esters (PMA) was examined since phosphorylation is a common mechanism of regulating the catalytic activity of many eukaryotic: Metabolic labelling, with [32 P]orthophosphate, of 20 HEK293 cells stably over-expressing hSK1 1000-fold revealed that hSK1 is, indeed, phosphorylated, and that this phosphorylation increases rapidly in response to cell exposure to TNF α (Fig 1) and PMA. Furthermore, this increase in hSK1 phosphorylation is highly correlated with the observed increase in sphingosine kinase activity in these cells 25 in response to TNF α (Fig 1) and PMA. These observations suggested that phosphorylation plays a role in hSK1 activation. Further evidence for this was also obtained using a hSK1 mutant defective in its ability to bind TRAF2. This hSK1 mutant was is neither activated (Xia *et al.* 2002) or phosphorylated in response to TNF α (Fig 2). PMA, however, induces both activation (Xia *et al.* 2002)and phosphorylation of this mutant (Fig 2).

30 *hSK1 is phosphorylated at Ser²²⁵*

Analysis of the hSK1 sequence with the NetPhos phosphorylation site prediction program (Blom *et al.* 1999) allowed the identification of several possible phosphorylation sites in hSK1 that were also conserved in the mouse and monkey SK1 isoforms (Fig 3). Alanine mutagenesis of these possible sites revealed that the hSK1 mutant containing the $\text{Ser}^{225} \rightarrow$ 5 Ala mutation was not phosphorylated (Fig 4). This suggested Ser^{225} as the sole physiologic phosphorylation site in hSK1. Further mutagenesis to alanine of Ser^{220} and Thr^{222} , two potentially phosphorylatable amino acids in the region around Ser^{225} had no effect on hSK1 phosphorylation (Fig 5). This indicated that the $\text{Ser}^{225} \rightarrow \text{Ala}$ mutation directly blocks phosphorylation at Ser^{225} rather than having a secondary effect on phosphorylation 10 at an adjacent site (i.e. Ser^{220} or Thr^{222}).

Activation of hSK1 requires phosphorylation at Ser^{225}

15 The hSK $^{\text{S}225\text{A}}$ mutant, when expressed in HEK293 cells, cannot be activated by cell treatment with TNF α or PMA (Fig 6). This provides strong evidence that phosphorylation Ser^{225} is essential for hSK1 activation.

ERK1, ERK2, and CDK2 specifically phosphorylate hSK1 at Ser^{225} in vitro

20 The amino acid sequence around Ser^{225} is SKTPAS 225 PVVVQ SEQ ID NO:3. The presence of a proline immediately *C*-terminal to Ser^{225} suggests a member of the proline-directed protein kinase family is responsible for hSK1 phosphorylation. In particular, the PASP sequence of this region is reminiscent of the ERK1/2 substrate recognition motif, PxS/TP, where x represents a small, neutral amino acid (Chen *et al.* 2001). Experiments 25 examining *in vitro* phosphorylation of purified recombinant hSK1 with ERK1, ERK2 and cyclin-dependent kinase 2 (CDK2) showed that all three kinases can phosphorylate hSK1 (Fig 7). Of these kinases, however, ERK2 showed the greatest efficiency of hSK1 phosphorylation. Further *in vitro* phosphorylation analysis with purified recombinant hSK $^{\text{S}225\text{A}}$ mutant showed that ERK2 could not phosphorylate this protein (Fig 8). ERK2 30 was, however, still able to phosphorylate recombinant hSK1 mutants containing alanine

mutations the only other potential proline-dependent kinase phosphorylation sites in hSK1 (Ser¹⁴⁸ and Thr²²²). This indicated that ERK2 specifically phosphorylates hSK1 at Ser²²⁵.

Increases in hSK1 phosphorylation in cells are blocked by a ERK1/2 pathway inhibitor,

5 *but not a CDK inhibitor*

The *in vivo* phosphorylation of hSK1 in HEK293 cells in response to TNF α and PMA in the presence of PD98059 (a chemical inhibitor of the ERK1/2 pathway) and Olomoucine (a chemical inhibitor of CDKs) was examined. The results (Fig 9) show that PD98059

10 prevents TNF α - and PMA-induced increases in hSK1 phosphorylation. In contrast, the presence of Olomoucine does not block PMA-induced hSK1 phosphorylation, and actually appears to increase basal hSK1 phosphorylation in the absence of agonist stimulation. This data suggests the ERK1/2 pathway is necessary for hSK1 phosphorylation.

15 Observations that ERK1/2 can phosphorylate hSK1 *in vitro*, and inhibitors of ERK1/2 pathway block hSK1 phosphorylation and activation in cells, both suggest that ERK1/2 directly phosphorylate hSK1 *in vivo*. Co-immunoprecipitation of ERK1/2 with hSK1 (Fig 9B) further confirm this by showing that hSK1 and ERK1/2 form a physical interaction in cells and provides evidence that hSK1 is an *in vivo* substrate for, and is directly activated.

20 by ERK1/2. Interaction of hSK1 with TRAF2 is essential for TNF α -induced hSK1 activation (Xia et al., 2002). One possibility is that TRAF2 may mediate the interaction between hSK1 and ERK1/2 to facilitate hSK1 phosphorylation and activation. This appears unlikely, however, since a previously described hSK1 mutant (hSK1^{TB2}) deficient in its ability to bind TRAF2 (Xia et al., 2002) also formed a complex with ERK1/2 (Fig: 25 9B). Therefore, although the interaction of hSK1 with TRAF2 appears important for agonist-dependent activation of hSK1 by ERK1/2, the nature of its role remains elusive.

The role of phosphorylation in the mechanism of hSK1 activation

30 The ability to specifically phosphorylate recombinant hSK1 at Ser²²⁵ *in vitro* with ERK2 afforded us the opportunity to examine the direct effect of this phosphorylation on the

catalytic activity of hSK1. It has been found that phosphorylation of hSK1 resulted in a dramatic increase in its sphingosine kinase activity. Quantitation of ^{32}P incorporation into the recombinant hSK1 following this *in vitro* phosphorylation showed 42 % of the hSK1 protein became phosphorylated, signifying that phosphorylation of hSK1 at Ser²²⁵ results 5 in an approximate 14-fold increase in its catalytic activity (Fig. 10A). Substrate kinetic analysis demonstrated that phosphorylation essentially increased the turnover number (k_{cat}) of hSK1 (Table 4). In contrast, the phosphorylated hSK1 had only a slightly lower K_M value for ATP, and an unaltered K_M value for sphingosine, compared to the non-phosphorylated enzyme (Fig. 10B, 10C and Table 4). Thus, although it has previously 10 been shown that hSK1 possesses considerable intrinsic catalytic activity in the absence of post-translational modifications, phosphorylation of this enzyme at Ser²²⁵ directly results in a marked, 14-fold increase in its catalytic efficiency.

Mutagenesis of Ser²²⁵ → Glu does not create a constitutively activated hSK1

15 By virtue of their negative charge the acidic amino acids glutamate and aspartate can sometimes mimic phosphorylated amino acids and create a protein conformation resembling the activated state. However, a hSK1 mutant containing the Ser²²⁵ → Glu mutation had similar activity to the wild-type hSK1 (Fig 11). Therefore, unlike the case in 20 some other enzymes this mutation not create a constitutively activated hSK1.

Phospho-hSK1-specific polyclonal antibodies

The crude polyclonal antisera raised in rabbits against the phosphopeptide centred around Ser²²⁵ of hSK1 was highly reactive in ELISA towards the phosphopeptide, but also showed 5 similar reactivity to the non-phosphorylated peptide (Fig 13). However, following affinity chromatography using the non-phosphorylated peptide to remove reactivity to this peptide, the antiserum showed high specificity in ELISA to the phosphopeptide (Fig 13). Furthermore, high specificity was also shown for the phosphorylated hSK1 protein in 10 Western blots (Fig 14). In these Western blots, the antiserum showed reactivity towards ERK2 phosphorylated hSK1, but showed little reactivity towards the non-phosphorylated wildtype hSK1 (Fig 14) or the hSK1^{S225A} mutant (Fig 15). Thus, we have used this purified 15 antiserum to show TNF α and PMA-dependent increases in hSK1 phosphorylation (Fig 15).

15 *Phosphorylation/activation of hSK1 is responsible for its effects on proliferation and transformation*

It has previously been shown that overexpression of wild type hSK1 significantly 20 enhanced cell proliferation and resulted in cell transformation (Xia et al., 2000). These effects were shown to be dependent on the catalytic activity of hSK1. However, since this enzyme has considerable intrinsic catalytic activity (Pitson et al., 2000a) it was not clear 25 from these studies whether hSK1 activation played a role in these effects. Certainly, it appeared that activation of hSK1 may be important in Ras-mediated transformation (Xia et al., 2000), making it tempting to speculate that the activated form of hSK1 may also be the key factor in transformation resulting from hSK1 overexpression. Elucidation of the 30 activation mechanism of hSK1 afforded the ability to examine this further through the use of the non-activatable Ser²²⁵ → Ala mutant of hSK1. Consistent with previous findings (Xia et al., 2000), HEK293T cells overexpressing wild type hSK1 displayed enhanced proliferation and serum-independent growth (Fig. 16). In contrast, cells overexpressing the non-phosphorylatable hSK1 mutant, hSK1^{S225A} displayed no difference in proliferative rate or serum-dependence to the control cells transfected with empty vector (Fig. 16). These

marked differences in the observed proliferative effects of wild type hSK1 and hSK1^{S225A} were seen even though the expression levels of both proteins were similar in these cells, as were the cellular sphingosine kinase activities.

5 Similar results were also observed in cellular transformation assays performed by colony formation in soft agar. As previously reported NIH3T3 cells transfected with wild type hSK1 displayed considerable transforming activity over that seen with control cells (Xia et al., 2000). In contrast, however, cells transfected with hSK1^{S225A} had remarkably less transforming activity (Fig. 17). This is despite the cells expressing similar levels of the

10 transfected proteins and possessing similar overall sphingosine kinase activities. This data provides compelling evidence that it is the phosphorylated, activated form of hSK1 that is responsible for the transforming capacity of this enzyme. Interestingly, it was also found that hSK1^{S225A} blocked Ras-induced cell transformation (Fig. 17), providing further evidence for the involvement of hSK1 activation in this process. Since the hSK1-

15 transfected cells already possess a high level of sphingosine kinase activity, the effect of activation on cell transformation may not just be a consequence of the resultant increases in catalytic activity of this enzyme. Instead, it may also be related to an activation-induced change in subcellular localisation of the activated hSK1.

20 *Translocation of hSK1 to membranes and increased intracellular and extracellular sphingosine 1-phosphate levels depends on hSK1 phosphorylation.*

Recent reports suggest that activation of hSK1 by PMA or PDGF is accompanied by translocation of the enzyme from the cytosol to membrane fractions. To test whether this

25 translocation depends on agonist-dependent phosphorylation of hSK1 we measured the levels of both total and phosphorylated hSK1 in cytosolic and membrane fractions with and without stimulation by TNF α or PMA of HEK293T cells overexpressing either wild type hSK1 or the phosphorylation deficient hSK1^{S225A} mutant (Fig. 18a). Stimulation with PMA, or TNF α consistently induced an increase in membrane-associated wild type hSK1. In contrast the Ser²²⁵

→ Ala mutation completely blocked any increase in membrane-associated hSK1, confirming that phosphorylation at that site is required for the translocation event.

Phosphorylation is not only required for agonist-stimulated increases in enzyme activity, as 5 demonstrated above, but is also essential for the activation-dependent elevation in S1P, the second messenger produced by hSK1 (Fig. 18b). Overexpression of wild type hSK1 resulted in large increases in both intracellular and extracellular S1P over that of vector-transfected cells, even in the absence of stimulus. Stimulation of these cells with either TNF α or PMA, however, resulted in further increases in both intracellular and extracellular S1P. This 10 mirrored well the changes in S1P, at lower overall levels, in empty vector transfected cells. In contrast, mutation of the Ser²²⁵ phosphorylation site completely eliminated any agonist-dependent changes in either intracellular or extracellular S1P. In addition, compared with wild type hSK1 transfected cells, reduced basal S1P levels were observed in cells overexpressing the hSK1^{S225A} mutant. This was most marked for extracellular S1P, and was despite the wild 15 type hSK1 and hSK1^{S225A}-overexpressing cells possessing similar basal sphingosine kinase activities. Consistent with ERK1/2 being the activator(s) of endogenous as well as overexpressed hSK1, inhibition with the ERK1/2 pathway inhibitor U0126 blocked TNF α - and PMA-induced increases in intracellular and extracellular S1P in both vector transfected and wild type hSK1 transfected cells. The inability of this inhibitor to reduce S1P levels to 20 that of the hSK1^{S225A} mutant is explained by an incomplete block of basal hSK1 phosphorylation.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood 25 that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 3 Mutagenic oligonucleotides used for site-directed mutagenesis of hSK1

Mutation	Sequence
S148A	CGGCTGCTGGCGCCCATGAAC - SEQ ID NO:4
S181A	TGTGGACCTCGAGGCTGAGAAGTA - SEQ ID NO:5
Y184A	AGTGAGAAGGCTCGGCGCCTGGGGAG - SEQ ID NO:6
S220A	AAGAGTGGCGCCAAGACAC - SEQ ID NO:7
T222A	AAGAGTGGGATCCAAGGCGCCTGCCTCC - SEQ ID NO:8
S225A	AAGACACCTGCGCGCCCGTTGTG - SEQ ID NO:9
S225E	ACACCTGCCGAACCGGTTGTGGTC - SEQ ID NO:10
T250A	TCTCACTGGGCAGTGGTGC - SEQ ID NO:11

Table 4. Substrate kinetics of *in vitro* phosphorylated hSK1

	k_{cat} (s ⁻¹)	K_M (ATP) (μM)	K_M (Sphingosine) (μM)
hSK1	93 ± 15	81 ± 12	15 ± 4
phospho-hSK1	1265 ± 86	56 ± 8	13 ± 3

BIBLIOGRAPHY

Blom, N *et al.* (1999) *J Mol Biol* **294**, 1351-1362

Chen, Z *et al.* (2001) *Chem Rev* **101**, 2449-2476

Choi, OH *et al.* (1996) *Nature* **380**, 634-639

Cuvilliver, O *et al.* (1998) *J Biol Chem* **273**, 2910-2916

Desai, NN *et al.* (1992) *J Biol Chem* **267**, 23122-23128

Kleuser, B *et al.* (1998) *Cancer Res* **58**, 1817-1823

Mandala, SM *et al.* (2000) *Proc Natl Acad Sci USA* **97**, 7859-7964

Mattie, M *et al.* (1994) *J Biol Chem* **269**, 3181-3188

Melendez, A *et al.* (1998) *J Biol Chem* **273**, 9393-9402

Meyer zu Heringdorf, D *et al.* (1998) *EMBO J* **17**, 2830-2838

Meyer zu Heringdorf, D *et al.* (1999) *FEBS Lett* **461**, 217-221

Olivera, A & Spiegel, S. (1993) *Nature* **365**, 557-560

Pitson, SM *et al.* (2000) *Biochem J* **350**, 429-441

Pitson, SM *et al.* (2000) *J Biol Chem* **275**, 33945-33950

Rius, RA *et al.* (1997) *FEBS Lett* **417**, 173-176

- 53 -

Spiegel, S *et al.* (1998) *Ann NY Acad Sci* **845**, 11-18

Su, Y *et al.* (1994) *J Biol Chem* **269**, 16512-16517

Van Veldhoven, PP *et al.* (2000) *Biochim Biophys Acta* **1487**, 128-134

Xia, P *et al.* (1998) *Proc Natl Acad Sci USA* **95**, 14196-14201

Xia, P *et al.* (2002) *J Biol Chem*, in press

DATED this 21st day of January 2003.

Medvet Science Pty Ltd
by its Patent Attorneys
DAVIES COLLISON CAVE

- 1 -

SEQUENCE LISTING

<110> Medvet Science Pty Ltd

<120> A Method of Modulating Cellular Activity - V

<130> 2608078/TDO

<160> 12

<170> PatentIn version 3.1

<210> 1

<211> 10

<212> PRT

<213> mammalian

<400> 1

Lys Thr Pro Ala Ser Pro Val Val Val Gln

1 5 10

<210> 2

<211> 14

<212> PRT

<213> mammalian

<400> 2

Cys Gly Ser Lys Thr Pro Ala Ser Pro Val Val Val Gln Gln

1 5 10

- 2 -

<210> 3
<211> 11
<212> PRT
<213> mammalian

<400> 3

Ser Lys Thr Pro Ala Ser Pro Val Val Val Gln
1 5 10

<210> 4
<211> 21
<212> DNA
<213> mammalian

<400> 4
cggtctgctgg cgcccatgaa c

21

<210> 5
<211> 24
<212> DNA
<213> mammalian

<400> 5
tgtggacctc gaggctgaga agta

24

<210> 6
<211> 27
<212> DNA
<213> mammalian

<400> 6
agtgagaagg ctcggcgctt gggggag

27

- 3 -

<210> 7

<211> 20

<212> DNA

<213> mammalian

<400> 7

aagagtgggc gccaaagacac

20

<210> 8

<211> 28

<212> DNA

<213> mammalian

<400> 8

aagagtggga tccaaaggcgc ctgcctcc

28

<210> 9

<211> 24

<212> DNA

<213> mammalian

<400> 9

aagacacacctg cggcgcccg ttgtg

24

<210> 10

<211> 24

<212> DNA

<213> mammalian

<400> 10

acacacctgcccc aaccgggttgtt ggtc

24

- 4 -

<210> 11
<211> 19
<212> DNA
<213> mammalian

<400> 11
tctcaactggg cagtgggtgc

19

<210> 12
<211> 384
<212> PRT
<213> mammalian

<400> 12

Met Asp Pro Ala Gly Gly Pro Arg Gly Val Leu Pro Arg Pro Cys Arg
1 5 10 15

Val Leu Val Leu Leu Asn Pro Arg Gly Gly Lys Gly Lys Ala Leu Gln
20 25 30

Leu Phe Arg Ser His Val Gln Pro Leu Leu Ala Glu Ala Glu Ile Ser
35 40 45

Phe Thr Leu Met Leu Thr Glu Arg Arg Asn His Ala Arg Glu Leu Val
50 55 60

Arg Ser Glu Glu Leu Gly Arg Trp Asp Ala Leu Val Val Met Ser Gly
65 70 75 80

Asp Gly Leu Met His Glu Val Val Asn Gly Leu Met Glu Arg Pro Asp
85 90 95

Trp Glu Thr Ala Ile Gln Lys Pro Leu Cys Ser Leu Pro Ala Gly Ser
100 105 110

- 5 -

Gly Asn Ala Leu Ala Ala Ser Leu Asn His Tyr Ala Gly Tyr Glu Gln
115 120 125

Val Thr Asn Glu Asp Leu Leu Thr Asn Cys Thr Leu Leu Leu Cys Arg
130 135 140

Arg Leu Leu Ser Pro Met Asn Leu Leu Ser Leu His Thr Ala Ser Gly
145 150 155 160

Leu Arg Leu Phe Ser Val Leu Ser Leu Ala Trp Gly Phe Ile Ala Asp
165 170 175

Val Asp Leu Glu Ser Glu Lys Tyr Arg Arg Leu Gly Glu Met Arg Phe
180 185 190

Thr Leu Gly Thr Phe Leu Arg Leu Ala Ala Leu Arg Thr Tyr Arg Gly
195 200 205

Arg Leu Ala Tyr Leu Pro Val Gly Arg Val Gly Ser Lys Thr Pro Ala
210 215 220

Ser Pro Val Val Val Gln Gln Gly Pro Val Asp Ala His Leu Val Pro
225 230 235 240

Leu Glu Glu Pro Val Pro Ser His Trp Thr Val Val Pro Asp Glu Asp
245 250 255

Phe Val Leu Val Leu Ala Leu Leu His Ser His Leu Gly Ser Glu Met
260 265 270

Phe Ala Ala Pro Met Gly Arg Cys Ala Ala Gly Val Met His Leu Phe
275 280 285

Tyr Val Arg Ala Gly Val Ser Arg Ala Met Leu Leu Arg Leu Phe Leu
290 295 300

Ala Met Glu Lys Gly Arg His Met Glu Tyr Glu Cys Pro Tyr Leu Val
305 310 315 320

- 6 -

Tyr Val Pro Val Val Ala Phe Arg Leu Glu Pro Lys Asp Gly Lys Gly
325 330 335

Met Phe Ala Val Asp Gly Glu Leu Met Val Ser Glu Ala Val Gln Gly
340 345 350

Gln Val His Pro Asn Tyr Phe Trp Met Val Ser Gly Cys Val Glu Pro
355 360 365

Pro Pro Ser Trp Lys Pro Gln Gln Met Pro Pro Pro Glu Glu Pro Leu
370 375 380

Figure 1

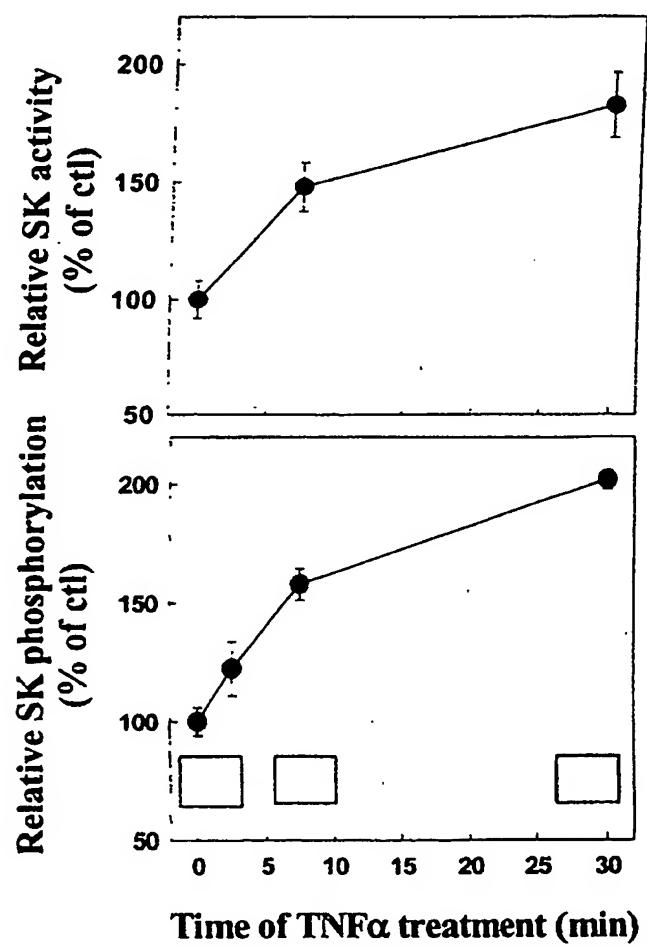


Figure 2

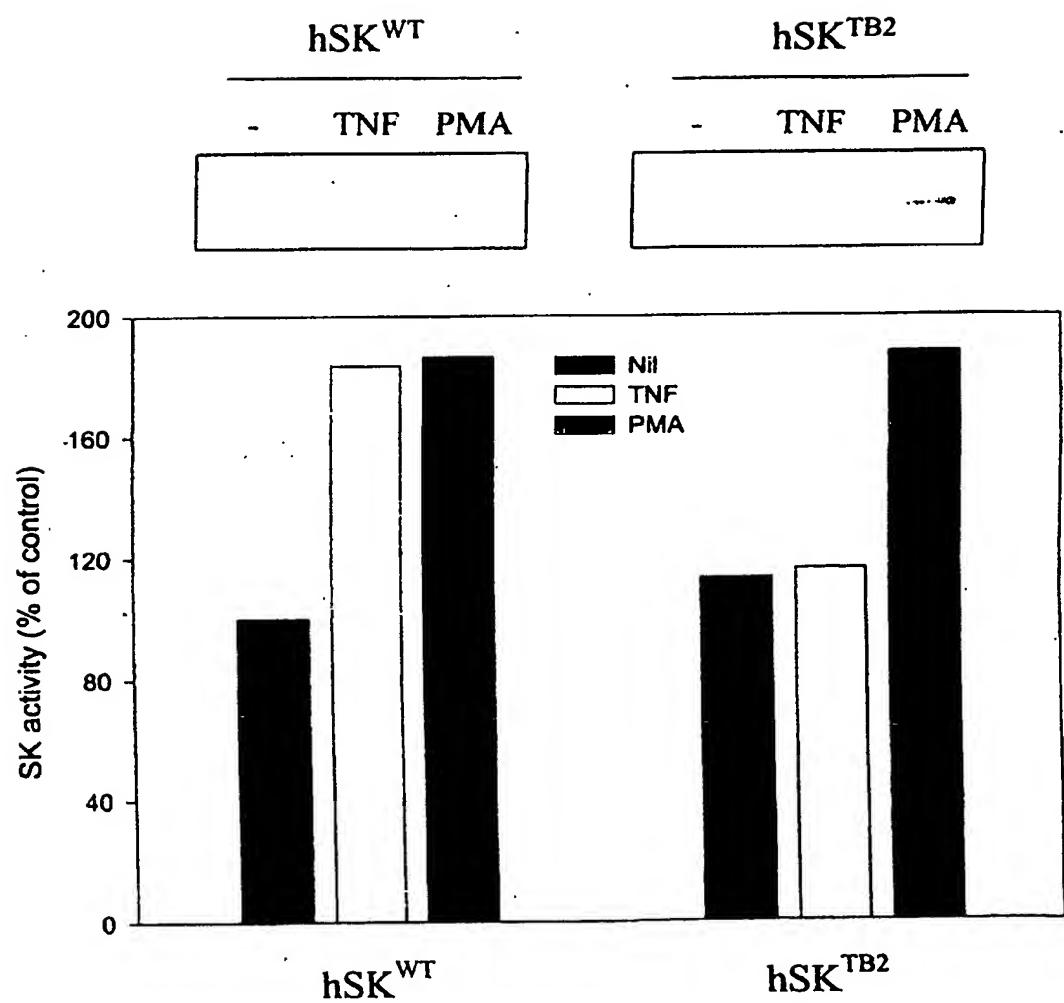


Figure 3

1 MDPAGGERGVLPRPCRVLVLLNPRGGKGKAIQLFRSHVQPILLAEAEISFTMLITERNHARELVRSEELG 70
71 RWDALVVMMSGDGLMHEVNGLMERPDWETAIQKPLCSLPAGSGNALLAASLNHYAGYEQVTNEDLLTNCTL 140
141 LICRRLQS^{SP}MNLLSLHTASGLRLFSVLSLAWGFIADVDL^{SE}KYR^YR^YGEMRFTLGTFLRLAALRTYRGR^L 210
211 AYLPVG^YGSKTP^{AS}PVVVQQGPVDAHLVPLEEPVPSH^{WT}YVPDED^YFLVLLHSHLGSEMFAAPMGR^C 280
281 AAGVMHILFYVRAGVSRAWILLRLFLAMEKGRHMEYECPYLVYVVPVVAFRLEPKDKGKGMFAUDGEIMVSEAV 350
351 QGQVHPNYFWMVSGCVERPPSWKPQQMPPPEEPL 384

Figure 4

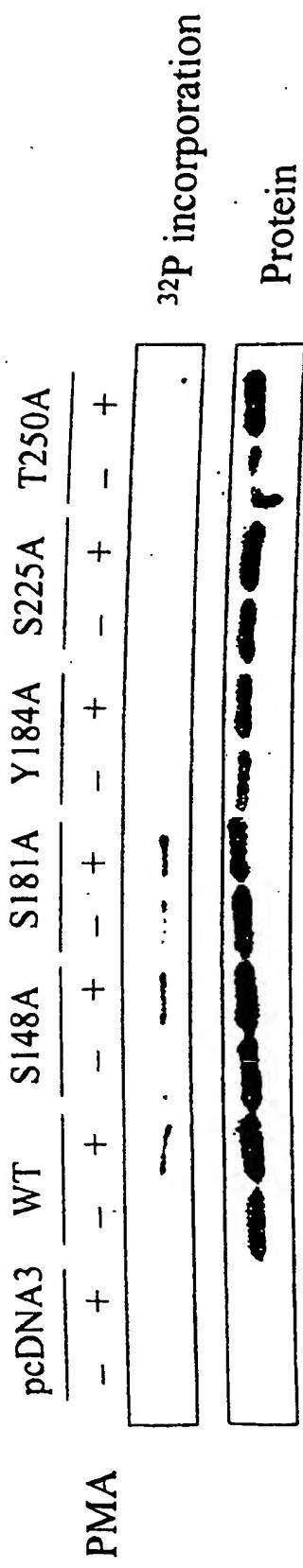


Figure 5

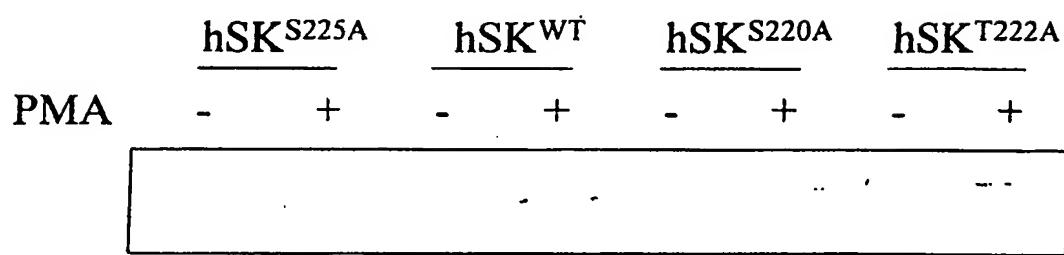


Figure 6

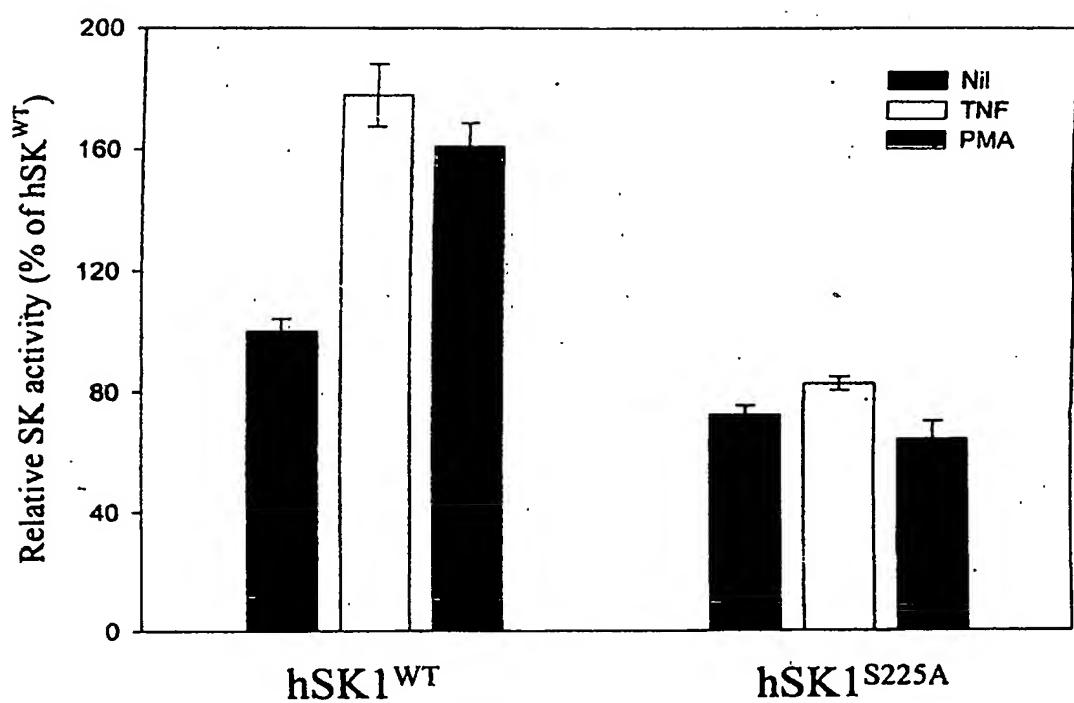


Figure 7

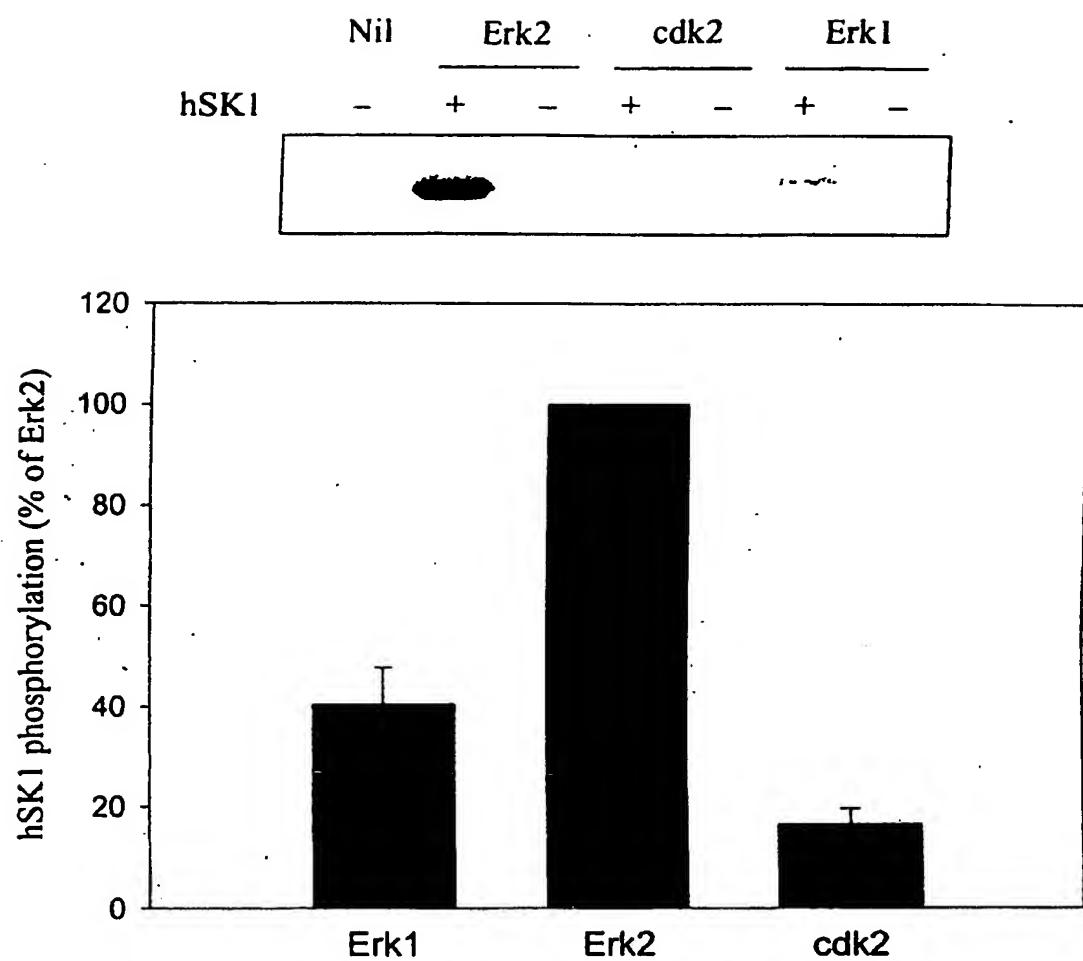


Figure 8

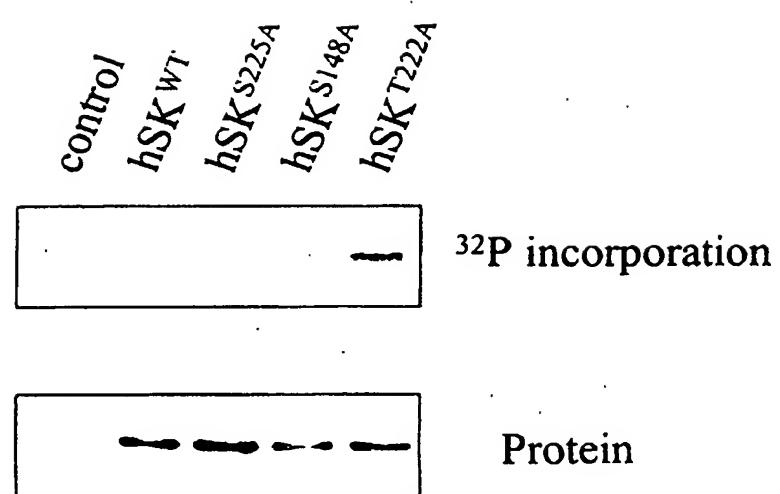


Figure 9A

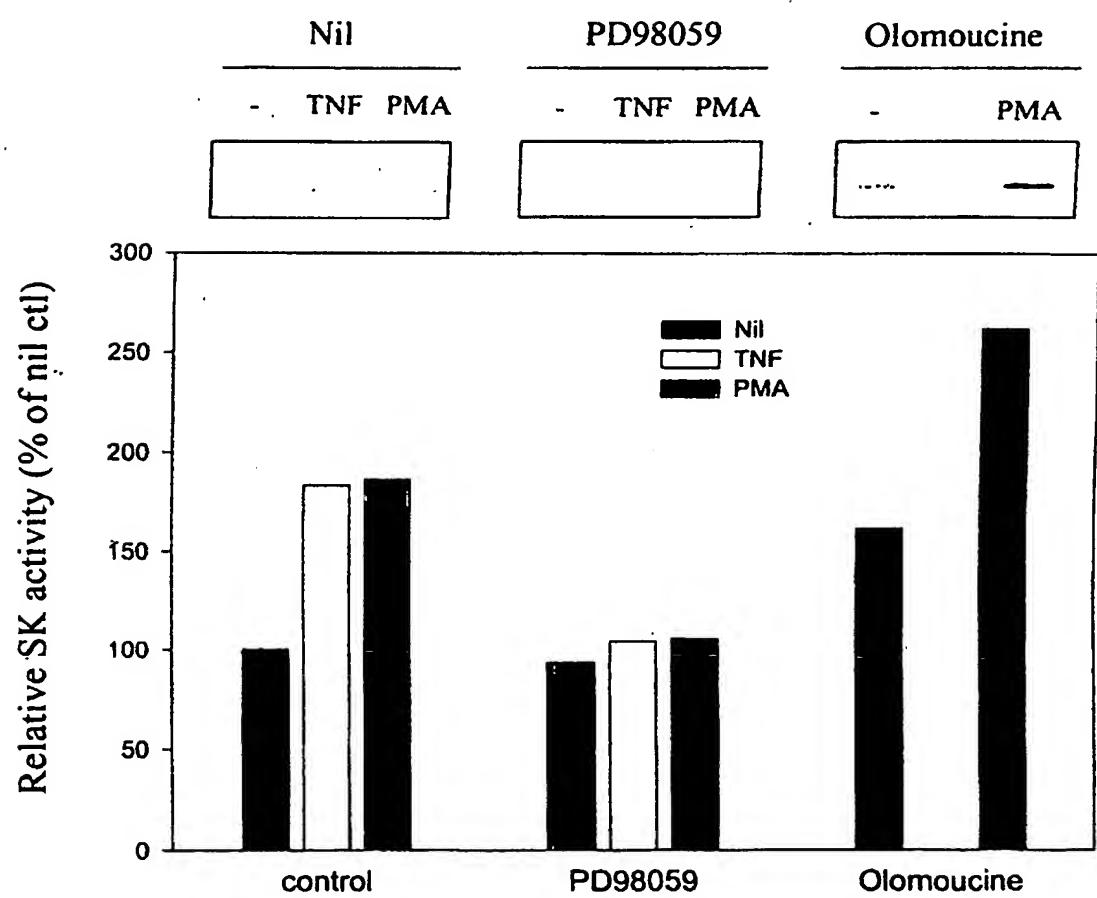


Figure 9B

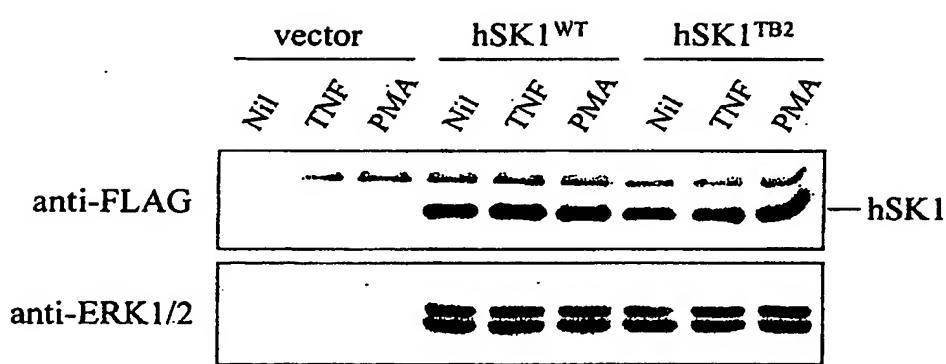


Figure 10A

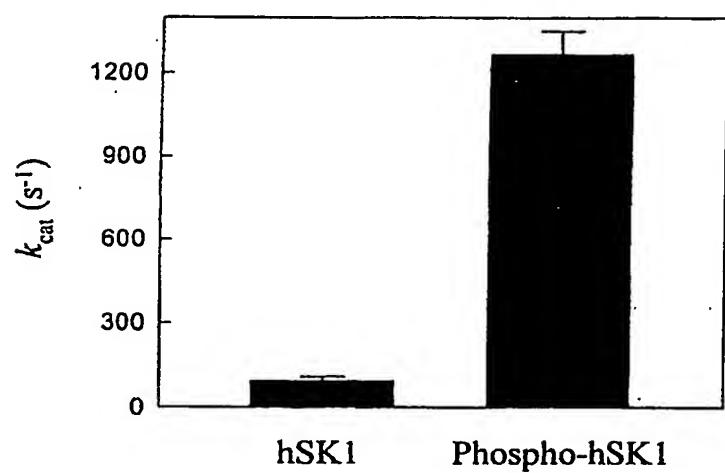


Figure 10B

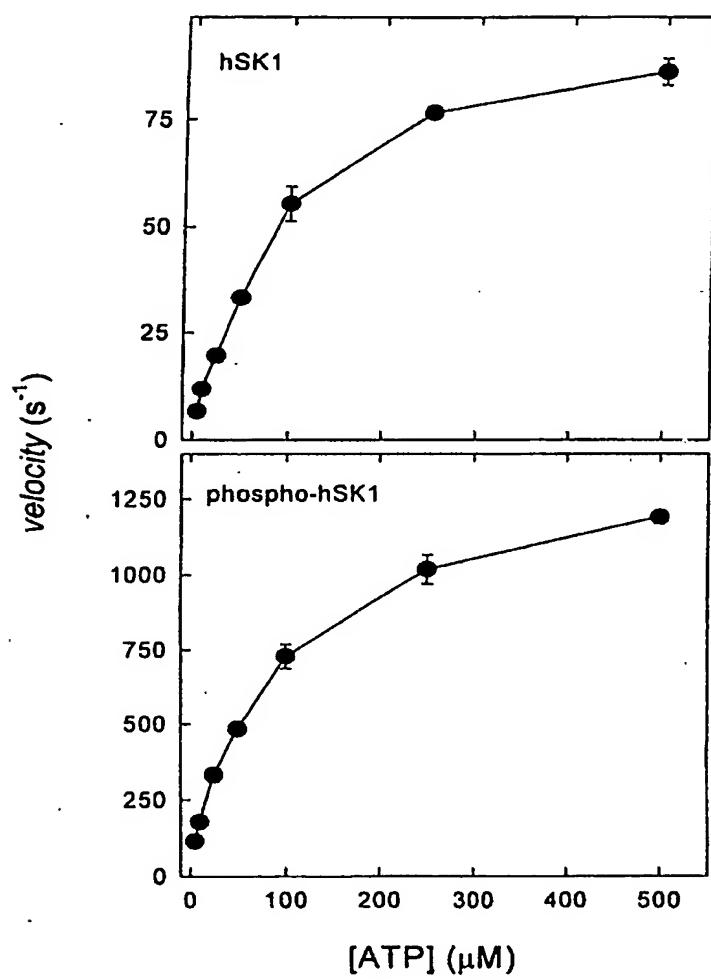


Figure 10C

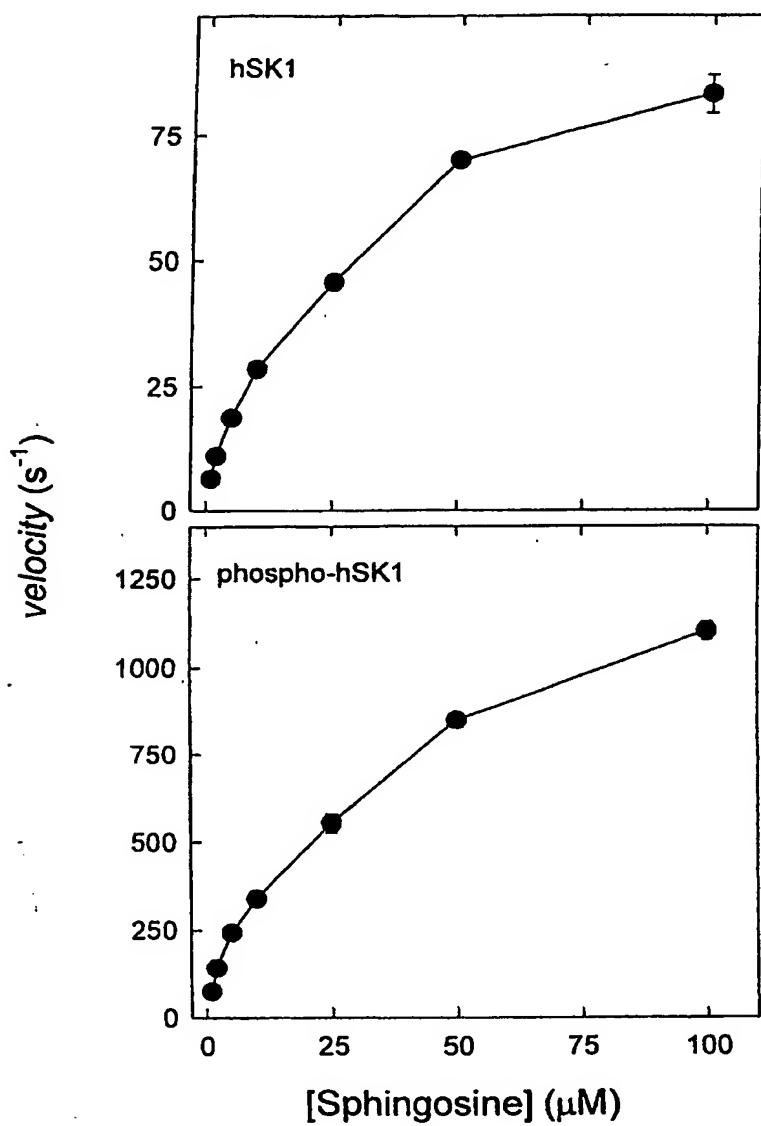


Figure 11

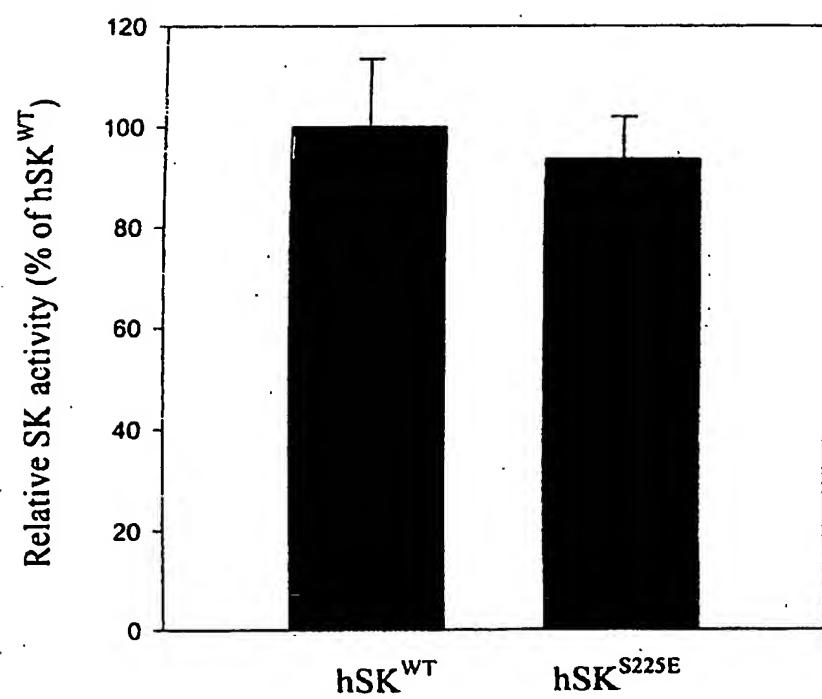


Figure 12

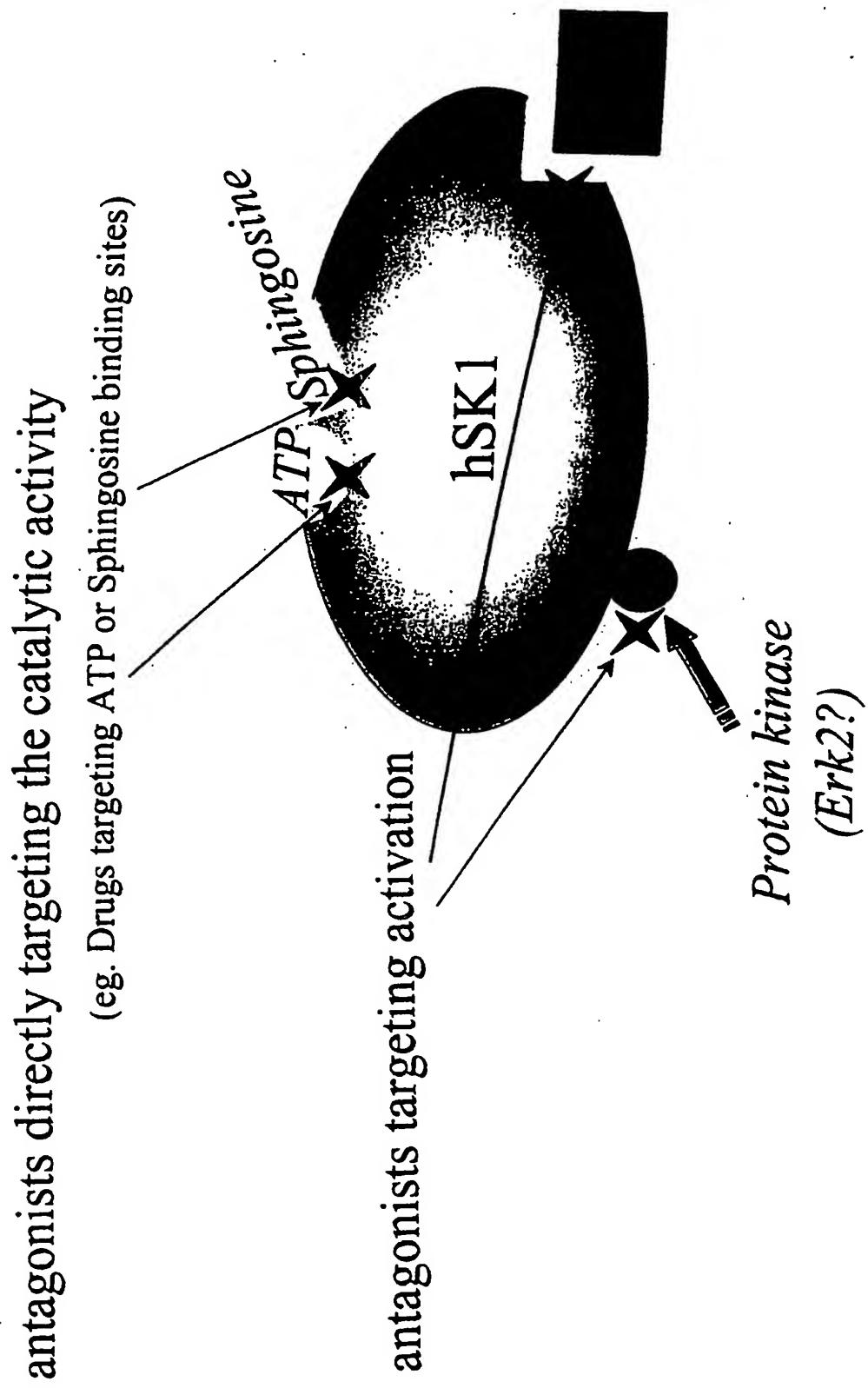


Figure 13

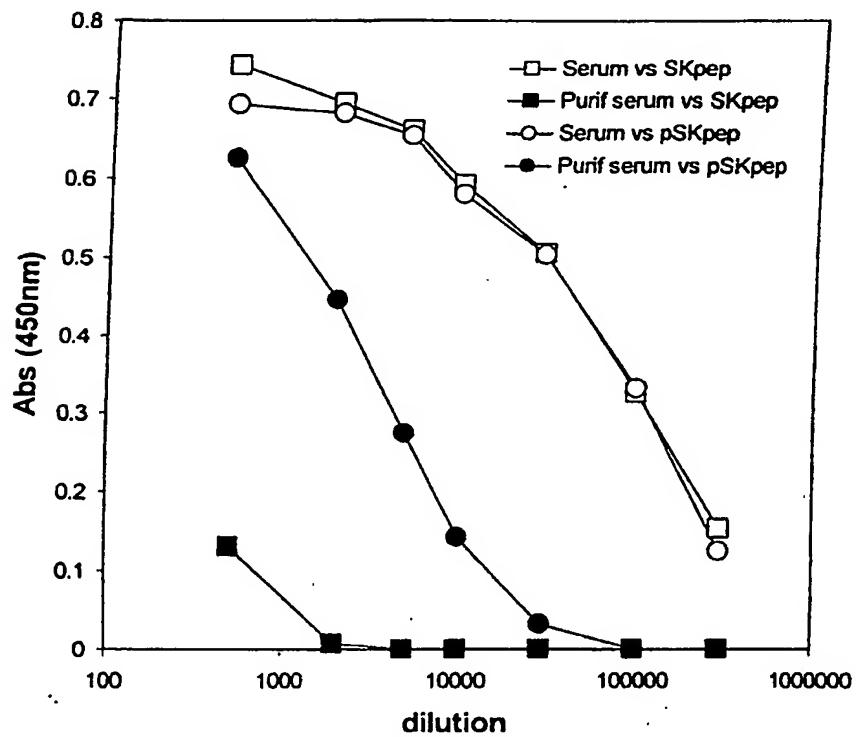


Figure 14

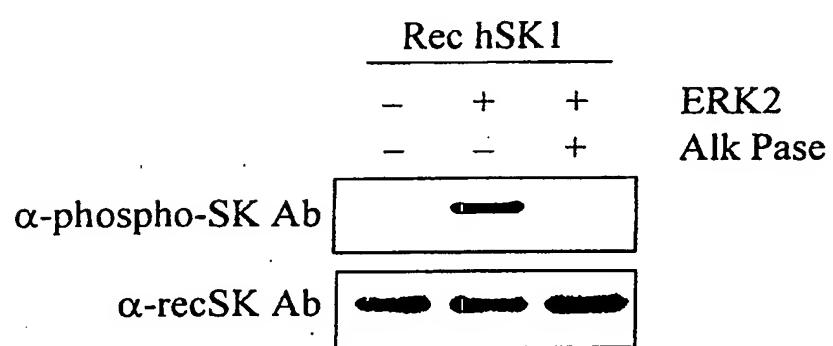


Figure 15

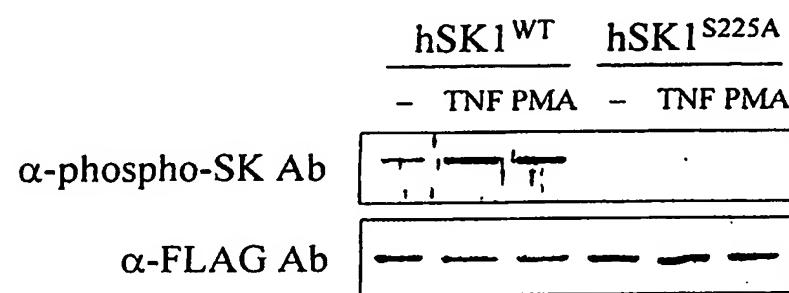


Figure 16

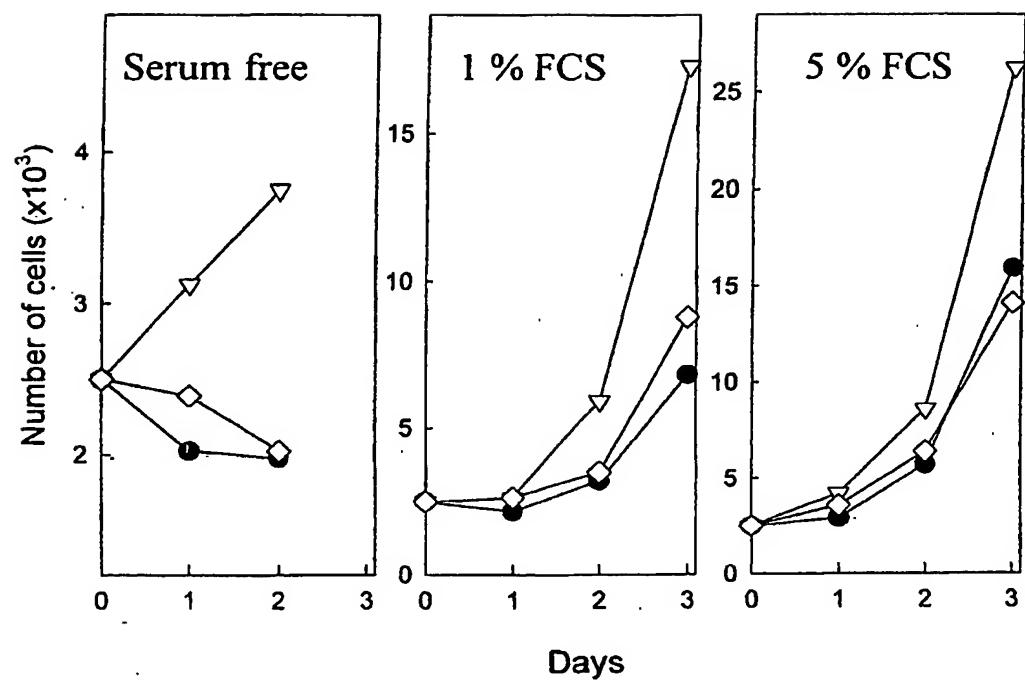


Figure 17

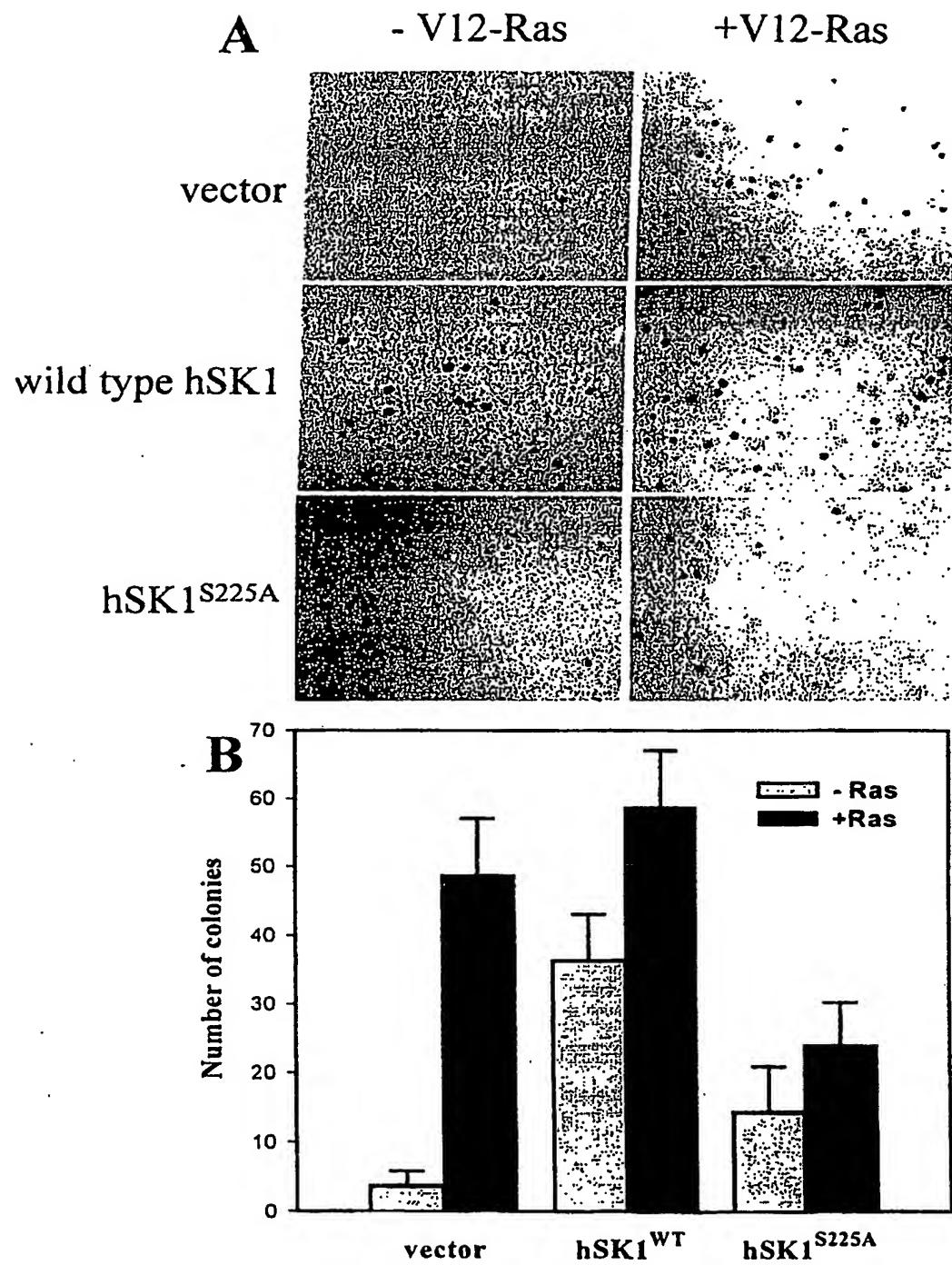
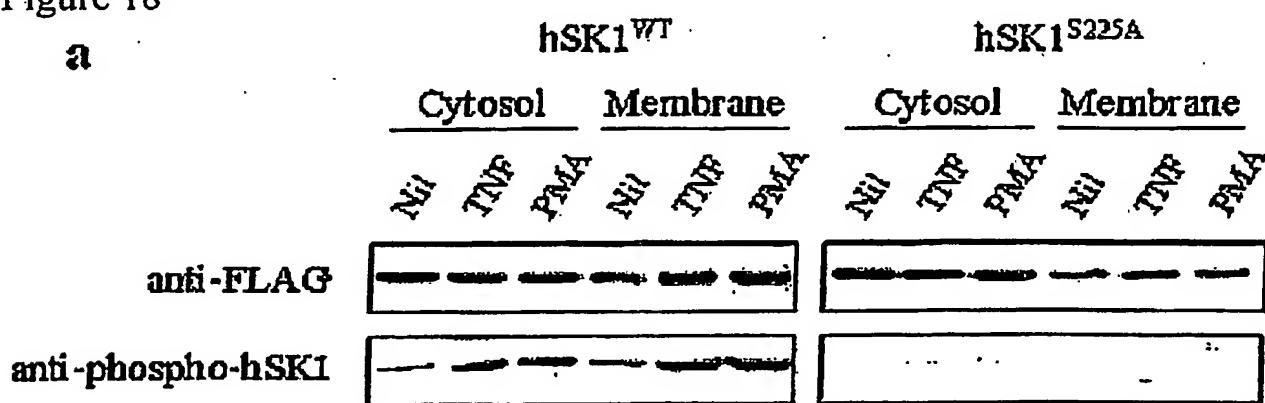


Figure 18

a



b

